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The Phospholipase C-IP_3 Pathway is Involved in Muscarinic Antinociception

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The cellular events involved in muscarinic analgesia were investigated in the mouse hot-plate test. Intracerebroventricular (i.c.v.) pretreatment with antisense oligonucleotides (aODNs) against the α subunit of G_q and G_{11} proteins prevented the analgesia induced by physostigmine and oxotremorine. Furthermore, administration of the phospholipase C (PLC) inhibitor U-73122, as well as the injection of an aODN complementary to the sequence of PLCβ_1, antagonized the increase of the pain threshold induced by both cholinomimetic drugs. In mice undergoing treatment with LiCl, which impairs phosphatidylinositol synthesis, or treatment with heparin, an IP_3 receptor antagonist, the antinociception induced by physostigmine and oxotremorine was dose-dependently antagonized. I.c.v. pretreatment with TMB-8, a blocker of Ca^{2+} release from intracellular stores, prevented the increase of pain threshold induced by the investigated cholinomimetic drugs. Coadministration of Ca^{2+} restored the muscarinic analgesia in LiCl, heparin, and TMB-8-preatreated mice. On the other hand, i.c.v. pretreatment with the selective protein kinase C (PKC) inhibitor calphostin C, resulted in a dose-dependent enhancement of physostigmine- and oxotremorine-induced antinociception. The administration of PKC activators, such as PMA and PDBu, dose dependently prevented the cholinomimetic drug-induced increase of pain threshold. Neither aODNs nor pharmacological treatments employed produced any behavioral impairment of mice as revealed by the rota-rod and hole-board tests. These results indicate a role for the PLC-IP_3 pathway in central muscarinic analgesia in mice. Furthermore, activation of PKC by cholinomimetic drugs may represent a pathway of negative modulation of muscarinic antinociception.
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INTRODUCTION

Several reports have provided evidence for the critical involvement of the cholinergic system in pain inhibitory pathways. The first observation that the cholinesterase inhibitor physostigmine increased the pain threshold in man was made more than 60 years ago (Pellandra, 1933). Since then, a vast literature has appeared describing the antinociceptive action of both cholinesterase inhibitors and cholinomimetic drugs (Hartvig *et al*, 1989).

The five types of mammalian muscarinic receptors, m1–m5, differ in primary structure as determined by molecular cloning (Caulfield, 1993). The molecularly defined m1, m2, and m3 receptors correlate with the pharmacologically defined binding M_1, M_2, and M_3 sites in mammalian tissues (Caulfield, 1993). It is well established that 'odd-numbered' muscarinic receptors (M_1–M_3–M_5) typically couple via the α subunits of the $G_{q/11}$ family to activate phospholipase C (PLC), stimulating phosphoinositide (PI) hydrolysis (Caulfield and Birdsall, 1998). In particular, reconstitution experiments with purified m1 receptors, G protein subunits, and PLC suggested that the β_1 subtype of PLC serves as the primary effector for the m1 receptor (Felder, 1995). Receptor-mediated activation of PLC results in the generation of at least two messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglicerol (DAG). The main effect of DAG is to activate protein kinase C (PKC); the effect of IP_3 is to release Ca^{2+} stored in the endoplasmic reticulum. The 'even-numbered' members (M_2–M_4) are preferentially coupled via G_i proteins to inhibit adenylate cyclase (Caulfield and Birdsall, 1998). Expression studies revealed that the cloned m2 and m4 receptors also stimulate PLC, although with lower efficiency than the PLC stimulation observed by m1 or m3 receptors (Ashkenazi *et al*, 1987).

Several literature reports indicate that cholinergic antinociception induced both directly, through muscarinic agonists, and indirectly, by enhancing ACh extracellular levels through cholinesterase inhibitors, is mediated by M_1 receptor stimulation, evidencing that M_1 muscarinic receptor subtype plays an essential role in the modulation of pain perception (Bartolini *et al*, 1992; Iwamoto and Marion, 1993; Ghelardini *et al*, 1996; Naguib and Yaksh, 1997; Ghelardini *et al*, 2000). The involvement of the M_2

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muscarinic receptor subtype in the induction of analgesia has also been postulated by using pharmacological antagonists (Gillbert *et al*, 1989) and by generating M_2 muscarinic receptor knockout mice (Gomeza *et al*, 2001).

Even if the receptor subtype involved in muscarinc antinociception has been widely investigated, the underlying intracellular mechanisms of muscarinic receptor-mediated increase of pain threshold are not elucidated. Since not only the 'odd-numbered', but also the 'even-numbered' muscarinic receptors activate PLC, the aim of the present study was to determine whether the stimulatory effect of cholinomimetics on PLC, and on DAG- and IP_3-mediated intracellular pathways, participates in the mechanism of central antinociception following activation of the muscarinic system.

METHODS

Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. A total of 15 mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at $23 \pm 1^{\circ}$C with a 12 h light/dark cycle, light at 7 am All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Hot-Plate Test

The method adopted was described by O'Callaghan and Holtzman (1975). Mice were placed inside a stainless-steel container, which was set thermostatically at $52.5 \pm 0.1^{\circ}$C in a precision water bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s) were measured with a stopwatch before and 15, 30, 45, and 60 min after administration of the analgesic drugs. The end point used was the licking of the fore or hind paws. Those mice scoring less than 12 s and more than 18 s in the pretest were rejected (30%). An arbitrary cutoff time of 45 s was adopted.

Rota-Rod Test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught *et al*. (1985). Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30, and 45 min after the beginning of the test.

Hole-Board Test

The hole-board test consisted of a 40 cm^2 plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed 4×4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (spontaneous motility). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

Intracerebroventricular (i.c.v.) Injection Technique

I.c.v. administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick (1957). During anesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl solution was then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of diluted 1 : 10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

Drugs

The following drugs were used: physostigmine hemisulphate, TMB-8 (8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate) hydrochloride, heparin sodium salt (Mol. Wt: approx. 60 000), lithium chloride, (Sigma, Milan, Italy); oxotremorine methiodide (R.B.I., Milan, Italy); calphostin C, U-73122 (1-[6-((17β-3-methoxyestra-4,3,5(10)-trien-17-yl)amino)hexyl]1H-pyrrole-2,5-dione), phorbol-12,13-dibutyrate (PDBu), phorbol-12-myristate-13-acetate (PMA), (Calbiochem, Milan Italy); D-amphetamine (De Angeli, Rome, Italy). Other chemicals were of the highest quality commercially available. U-73122, calphostin C, PMA, and PDBu were dissolved in 0.5% DMSO, whereas all other drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse by i.c.v. injection and 10 ml kg^{-1} by subcutaneous (s.c.) injection. PMA, PDBu, and Calphostin C were injected i.c.v. 1 h before the test; LiCl was administered s.c. 18 h before the test; U-73122, TMB-8, heparin, Ca^{2+} were injected 10 min before behavioral tests. Doses and administration schedule were chosen on the basis of time-course and dose–response experiments previously performed in our laboratory.

Furthermore, literature data confirm the selectivity and efficacy of the above-mentioned treatments at time and concentration used.

Antisense oligonucleotides (ODNs). Phosphodiester ODNs protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Tib-Molbiol (Genoa, Italy). The sequences are the following: anti-$G_{q\alpha}$: 5′-C*G*G CTA CAC GGT CCA AGT C*A*T-3′, corresponding to nucleotides 484–504 of the $G_q\alpha$ sequence; anti-$G_{11\alpha}$: 5′-C*T*G TGG CGA TGC GGT CCA C*G*T-3′, corresponding to nucleotides 487–507 of the $G_{11}\alpha$ sequence; anti-PLCβ_1: 5′-G*C*T GTC GGA CAC G*C*A-3′, corresponding to nucleotides 49–63 of the PLCβ_1 gene sequence. All aODNs were previously characterized by *in vitro*‘ and *in vivo* experiments (Sanchez-Blazquez and Garzon, 1998). We also confirmed the aODN effect on Gα and PLCβ1 protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of $G_{q\alpha}$(36.4 $\pm$ 10.6), $G_{11\alpha}$ (38.9 $\pm$ 13.6), and PLCβ_1 (45.1 $\pm$ 6.9) after aODN treatment in comparison with mice treated with degenerated oligonucleotide (dODN). A 21-mer fully dODN 5′-N*N*N NNN NNN NNN NNN N*N*N-3′ (where N is G, or C, or A, or T) and a 15-mer fully dODN 5′-N*N*N NNN NNN NNN N*N *N-3′ (where N is G, or C, or A, or T) were used as a control, respectively, for the 21-mer anti-$G_q\alpha$ and anti-$G_{11}\alpha$, and for the 15-mer anti-PLCβ_1. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma, Italy) to enhance both uptake and stability. aODN or dODN were preincubated at 37°C for 30 min with 13 μM DOTAP and supplied to mice by i.c.v. injection of 5 μl solution 72, 48, and 24 h prior to the behavioral tests.

Statistical Analysis

All experimental results are given as the mean $\pm$ SEM. An analysis of variance (ANOVA), followed by Fisher's protected least significant difference procedure for *post hoc* comparison, were used to verify significance between two means of behavioral results. Data were analyzed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

RESULTS

Role of $G_{q\alpha}$ and $G_{11\alpha}$ on Muscarinic Antinociception

The effect of pretreatment with antisense ODNs (aODNs) to the α subunit of G_q and G_{11} proteins on physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg kg^{-1} s.c.)-induced antinociception was evaluated in the mouse hot-plate test. Anti-$G_{q\alpha}$ (2–3 nmol per mouse i.c.v.) antagonized the analgesia induced by physostigmine and oxotremorine, whereas the anti-$G_{11\alpha}$ (3 nmol per mouse i.c.v.) significantly reduced the increase of the pain threshold produced by both cholinomimetic drugs (Figure 1). The aODNs, when injected alone, did not modify the pain threshold in comparison with control animals. Furthermore, the i.c.v. injection of dODN (3 nmol per mouse i.c.v.), used as control, neither modified the licking latency of mice nor altered the sensitivity to analgesic drugs in comparison with naive and saline-treated mice (data not shown).

Role of PLCβ_1 on Muscarinic Antinociception

The administration of the PLC inhibitor U-73122 (1.25–5.0 μg per mouse i.c.v.) dose-dependently prevented physostigmine (0.1 mg kg^{-1} s.c.) antinociception. The dose of

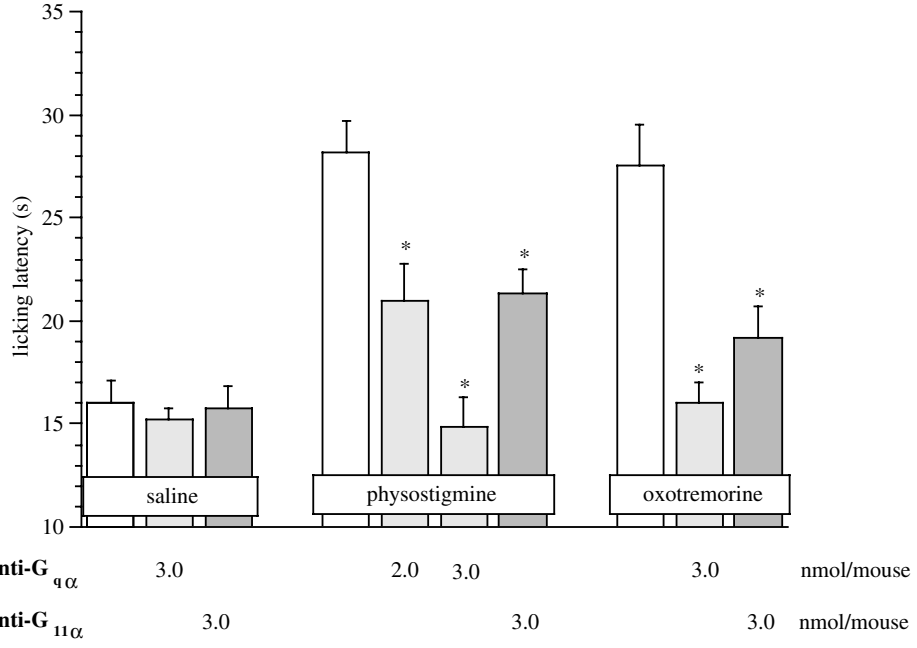

Figure 1 Prevention by pretreatment with anti-$G_{q\alpha}$ (2–3 nmol per mouse i.c.v.) and anti-$G_{11\alpha}$ (3 nmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after cholinomimetics administration. ODNs were administered 72, 48, and 24 h prior to the behavioral tests. Vertical lines represent SEM. *$P < 0.05$ in comparison with corresponding analgesic drug-treated mice.

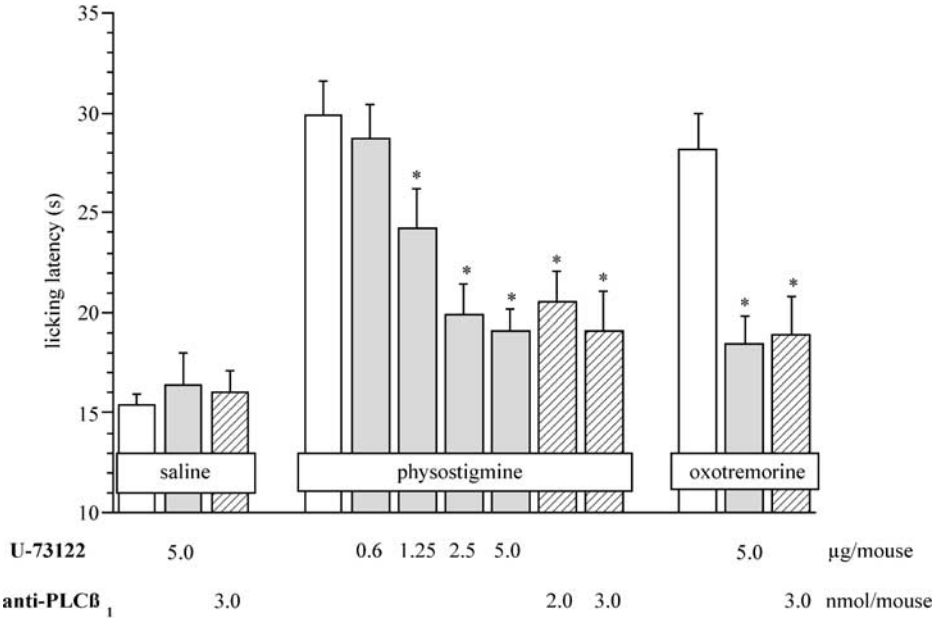

Figure 2 Prevention by pretreatment with U-73122 (0.6–5 μg per mouse i.c.v.) and anti-PLCβ_1 (2–3 nmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after cholinomimetics administration. ODNs were administered 72, 48, and 24 h prior to the behavioral tests. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice.

0.6 μg per mouse i.c.v. was devoid of any effect, whereas the maximum antagonistic effect was reached at 5.0 μg per mouse i.c.v. (Figure 2). Similarly, oxotremorine (60 μg kg^{-1} s.c.) antinociception was prevented by U-73122 (5.0 μg per mouse i.c.v.) administration (Figure 2). Pretreatment with an anti-PLCβ_1 (2–3 nmol per mouse i.c.v.) prevented the increase of the pain threshold induced by both physostigmine and oxotremorine (Figure 2). U-73122 and anti-PLCβ_1, when injected alone, produced neither an hyperalgesic nor an analgesic effect (Figure 2). The i.c.v. injection of dODN (3 nmol per mouse i.c.v.), used as control, neither modified the licking latency of mice nor altered the sensitivity to analgesic drugs in comparison with naive and saline-treated mice (data not shown).

Role of IP$_3$ on Muscarinic Antinociception

Pretreatment with LiCl, which impairs phosphatidylinositol synthesis, 18 h before the test dose-dependently reduced the antinociception induced by physostigmine (0.1 mg kg^{-1} s.c.). LiCl at 40 mg kg^{-1} s.c. was devoid of any effect. The dose of LiCl of 200 mg kg^{-1} s.c. reduced physostigmine analgesic effect without reaching the statistical significance whereas the dose of 400 mg kg^{-1} s.c. significantly prevented the increase of the pain threshold induced by the cholinesterase inhibitor. The maximum antagonistic effect was reached at 500 mg kg^{-1} s.c. The prevention of physostigmine antinociception induced by LiCl (500 mg kg^{-1} s.c.) was dose-dependently reverted by Ca^{2+} (0.05–0.1 μmol per mouse i.c.v.) (Figure 3). Similarly, LiCl (500 mg kg^{-1} s.c.) was also able to prevent oxotremorine (60 μg kg^{-1} s.c.) antinociception and the coadministration of Ca^{2+} (0.1 μmol per mouse i.c.v.) reverted the LiCl-induced effect (Figure 3). The i.c.v. injection of LiCl (500 mg kg^{-1} s.c.) and Ca^{2+} (0.1 μmol per mouse i.c.v.) alone did not modify the licking latency of mice in comparison with saline-treated animals (Figure 3).

The administration of heparin (1–40 μg per mouse i.c.v.), an antagonist of IP$_3$ receptors, prevented the increase of the pain threshold induced by physostigmine (0.1 mg kg^{-1} s.c.) in a dose-dependent manner, reaching its maximum effect at 40 μg per mouse i.c.v. (Figure 4a). Furthermore, heparin (20 μg per mouse i.c.v.) exerted its antagonistic effect at various doses of physostigmine (0.05–0.20 mg kg^{-1} s.c.) shifting to the right the physostigmine dose–response curve (Figure 4b). Heparin 40 μg per mouse i.c.v. was also able to prevent oxotremorine (60 μg kg^{-1} s.c.) antinociception without modifying the mouse pain threshold when injected alone (Figure 4a). Coadministration of Ca^{2+} (0.05–0.1 μmol per mouse i.c.v.) reverted the heparin-induced antagonistic effect (Figure 4a).

TMB-8, a blocker of Ca^{2+} release from intracellular stores, dose-dependently antagonized physostigmine (0.1 mg kg^{-1} s.c.) antinociception. The dose of TMB-8 of 0.01 μg per mouse i.c.v. reduced physostigmine analgesic effect without reaching statistical significance; the dose of 0.1 μg per mouse i.c.v significantly prevented the increase of the pain threshold induced by the cholinesterase inhibitor whereas the maximum antagonistic effect was reached at 1 μg per mouse i.c.v. (Figure 5a). The prevention of physostigmine antinociception induced by TMB-8 (1 μg per mouse i.c.v.) was dose-dependently reverted by Ca^{2+} (0.001–0.05 μmol per mouse i.c.v.). TMB-8 also shifted to the right the dose–response curve of physostigmine (0.05–0.2 mg kg^{-1} s.c.), as illustrated in Figure 5b. The antinociception induced by oxotremorine (60 μg kg^{-1} s.c.) was also antagonized by pretreatment with TMB-8 (1 μg per mouse i.c.v.); this effect was completely reverted by the coadministration of Ca^{2+} (0.05 μmol per mouse i.c.v.) (Figure 5a). TMB-8, when administered alone at the highest effective dose, did not

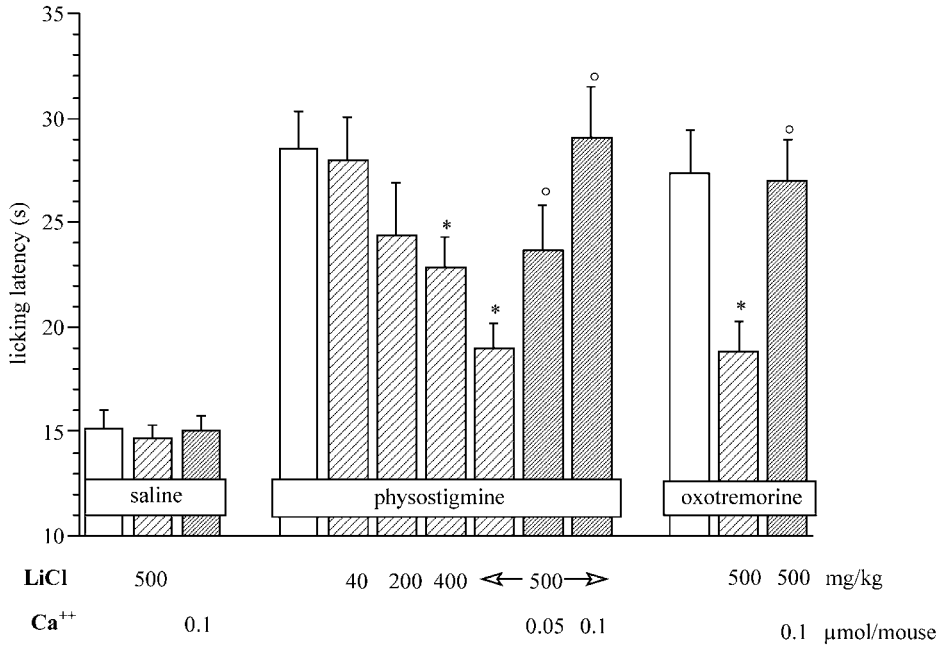

Figure 3 Prevention by pretreatment with LiCl (40–500 mg kg^{-1} s.c.) and reversal of the antagonism by Ca^{2+} (0.05–0.1 μmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after cholinomimetics administration. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice; °$P<0.05$ in comparison with saline-treated mice.

modify the licking latency values in comparison with control animals (Figure 5a).

Role of PKC on Muscarinic Antinociception

Pretreatment with the PKC blocker calphostin C produced a dose-dependent potentiation of physostigmine-induced (0.1 mg kg^{-1} s.c.) antinociception in the mouse hot-plate test. The maximum effect was obtained at a dose of 200 ng per mouse i.c.v., whereas the increase of the licking latency values produced by a dose of 30 ng per mouse i.c.v. was not statistically significant. A dose of 10 ng per mouse i.c.v. was devoid of any effect (Figure 6a). Furthermore, calphostin C (100 ng per mouse i.c.v.) exerted its potentiating effect at various doses of physostigmine (0.05–0.15 mg kg^{-1} s.c.), shifting to the left the dose–response curve of the cholinesterase inhibitor (Figure 6a). Similar to physostigmine, calphostin C (100 ng per mouse i.c.v.) also potentiated the increase of the pain threshold induced by oxotremorine (60 μg kg^{-1} s.c.), without modifying the mouse pain threshold when administered alone (Figure 6a).

The PKC activators PDBu (10–50 pmol per mouse i.c.v.) and PMA (1–15 pmol per mouse i.c.v.) dose-dependently antagonized the analgesia induced by both physostigmine (0.1 mg kg^{-1} s.c.) and oxotremorine (60 μg kg^{-1} s.c.) (Figure 7). The maximum antagonistic effect of PDBu and PMA was obtained, respectively, at the dose of 40 and 15 pmol per mouse i.c.v.; at the highest effective doses the two PKC activators did not modify the animals' licking latency values in comparison with control animals (Figure 7).

Effect of Treatments on Mouse Behavior

The compounds investigated, at the highest effective doses, were tested in order to assess their effect on mouse behavior. Mice pretreated with aODNs (3.0 nmol per mouse i.c.v.), dODN (3.0 nmol per mouse i.c.v.), LiCl (500 mg kg^{-1} s.c.), TMB-8 (1 μg per mouse i.c.v.), Ca^{2+} (0.1 μmol per mouse i.c.v.), heparin (40 μg per mouse i.c.v.), U-73122 (5 μg per mouse i.c.v.), calphostin C (100 ng per mouse i.c.v.), PMA (15 pmol per mouse i.c.v.), and PDBu (40 pmol per mouse i.c.v.) were evaluated for motor coordination by use of the rota-rod test, and for spontaneous motility and inspection activity by use of the hole-board test.

The endurance time, evaluated before and 15, 30, and 45 min after the beginning of the rota-rod test, showed the lack of any impairment in the motor coordination of animals pretreated with aODNs in comparison with the dODN group. Mice pretreated with LiCl, TMB-8, Ca^{2+}, heparin, or pretreated with U-73122, calphostin C, PMA, PDBu did not show any alteration of motor coordination in comparison, respectively, with saline or vehicle group (data not shown).

The spontaneous motility as well as the inspection activity of mice, expressed as counts in 10 min, was unmodified by pretreatment with aODNs in comparison with the dODN group, by pretreatment with LiCl, TMB-8, Ca^{2+}, heparin in comparison with saline group, or by pretreatment with U-73122, calphostin C, PMA, PDBu in comparison with vehicle group (Table 1).

Higher doses of the intracellular modulators employed were not investigated since they induced behavioral side effects such as tremors and convulsions.

DISCUSSION

The present study investigated and elucidated the intracellular mechanisms involved in muscarinic analgesia in a condition of acute thermal nociception in normal animals.

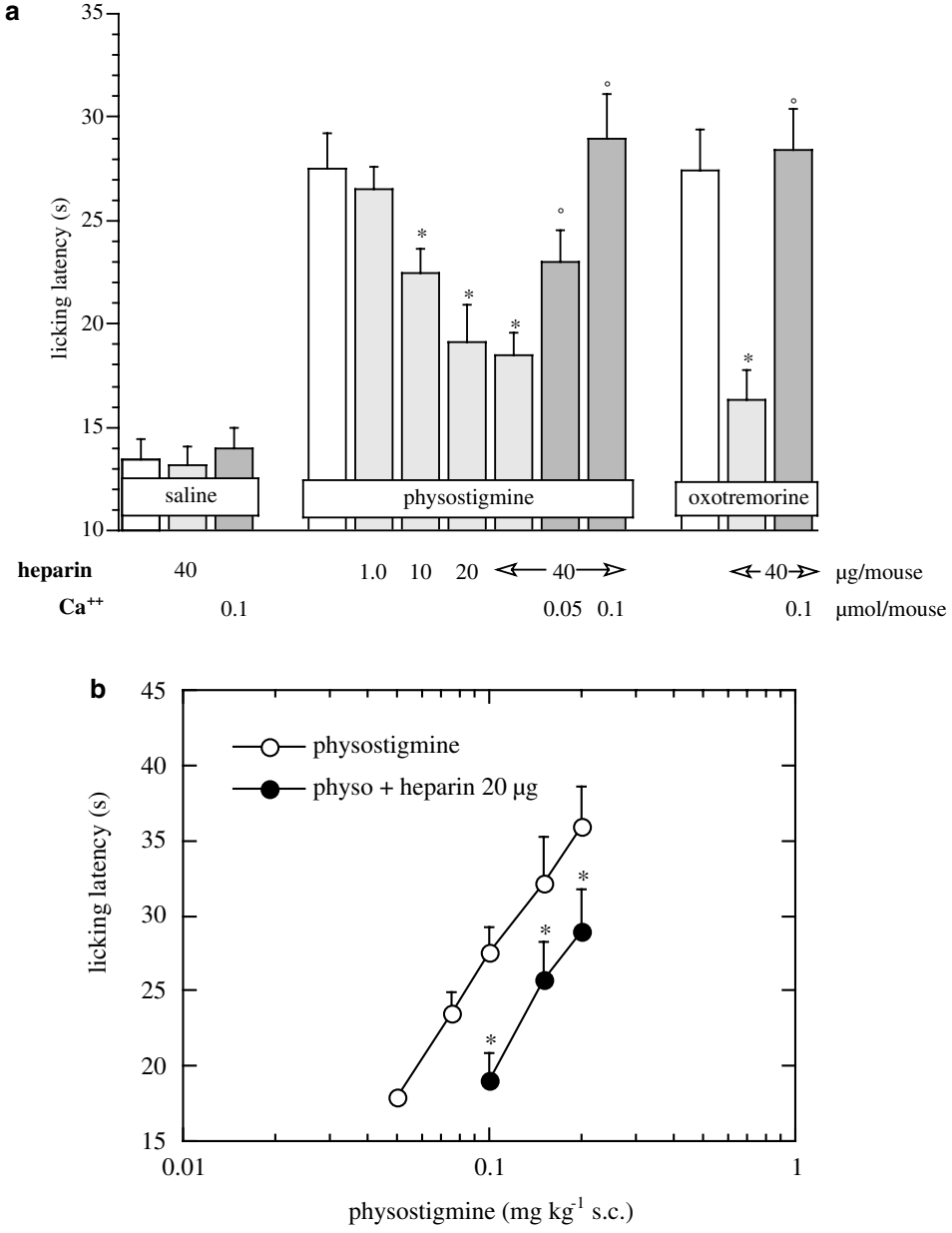

Figure 4 Effect of heparin on physostigmine and oxotremorine antinociception in the mouse hot-plate test. (a) Prevention by heparin (1–40 μg per mouse i.c.v.) and reversal of antagonism by Ca^{2+} (0.05–0.1 μmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice; °$P<0.05$ in comparison with saline-treated mice. (b) Shift to the right of the dose–response curve of physostigmine (0.05–0.2 mg kg^{-1} s.c.) by heparin (20 μg per mouse i.c.v.). Vertical lines represent SEM. *$P<0.05$ in comparison with physostigmine-treated mice.

The importance of the receptor-mediated activation of the PLC-IP$_3$ pathway to obtain an increase of the pain threshold was demonstrated.

The administration of U-73122, an aminosteroid characterized as an inhibitor of G protein-mediated PLC (Wakdo *et al*, 1983; Yule and Williams, 1992), dose-dependently prevented the antinociception induced by the cholinomimetic compounds physostigmine and oxotremorine. Phosphoinositide-specific PLC represents a family of isozymes found in eukaryotes composed by β, γ, and δ subtypes, which cleaves the polar head group from inositol lipids (Rebecchi and Pentyala, 2000). Among them, the isozyme PLCβ_1 has been reported to be activated selectively by M_1 receptors (Felder, 1995), a muscarinic receptor subtype mainly involved in the induction of analgesia (Ghelardini *et al*, 2000). In order to elucidate the role of this PLC subtype in muscarinic antinociception, an aODN complementary to the sequence of PLCβ_1 was employed. The inhibition of the expression of this isozyme prevented the increase of pain threshold induced by both cholinomimetic drugs used. These results indicate PLC, and in particular the isozyme PLCβ_1, as an important intracellular effector in muscarinic analgesia.

The 'odd-numbered' muscarinic receptors activate PLC via the α subunit of the $G_{q/11}$ proteins (Caulfield and Birdsall, 1998). Furthermore, activation of PLCβ_1 is achieved with the help of Gα subunits of the G_q proteins (Taylor *et al*, 1991). The administration of aODNs against

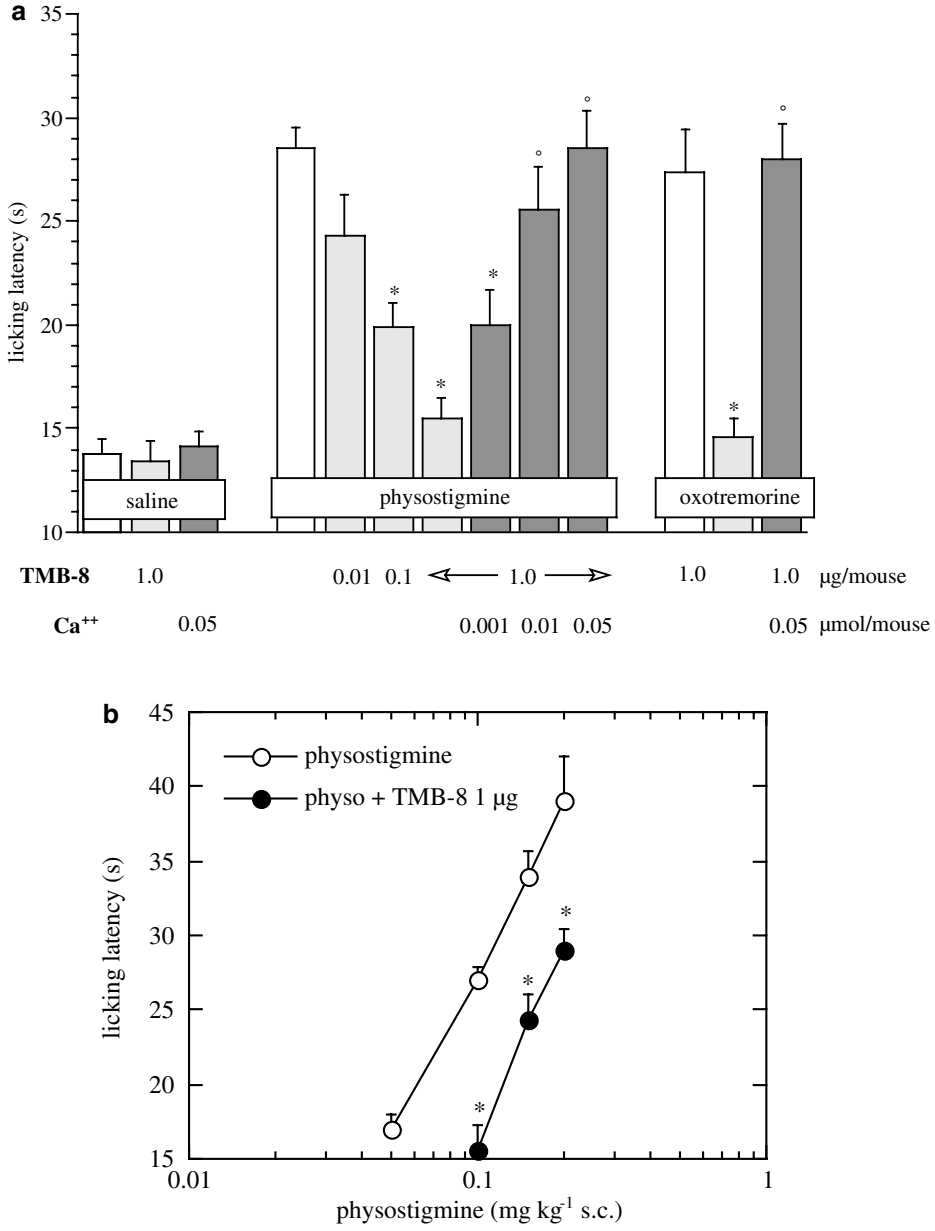

Figure 5 Effect of TMB-8 on physostigmine and oxotremorine antinociception in the mouse hot-plate test. (a) Prevention by TMB-8 (0.01–1 µg per mouse i.c.v.) and reversal of antagonism by Ca^{2+} (0.001–0.05 µmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 µg per mouse i.c.v.)-induced antinociception. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice; °$P<0.05$ in comparison with saline-treated mice. (b) Shift to the right of the dose–response curve of physostigmine (0.05–0.2 mg kg^{-1} s.c.) by TMB-8 (1 µg per mouse i.c.v.). Vertical lines represent SEM. *$P<0.05$ in comparison with physostigmine-treated mice.

the α subunit of G_q and G_{11} proteins antagonized the physostigmine and oxotremorine antinociception. These results indicate that the stimulation of the PLC-mediated intracellular pathway in muscarinic analgesia requires the receptor-mediated activation of $G_{q/11}$ transducer proteins.

PLC isozymes hydrolyze the highly phosphorylated lipid phosphatidylinositol 4,5-biphosphate generating two intracellular products: IP_3, a universal calcium-mobilizing second messenger, and DAG, an activator of PKC. To investigate the IP_3-mediated pathway, LiCl, an uncompetitive inhibitor of inositol monophosphatase, which regenerates inositol from inositol monophosphate, was used. The LiCl-induced inhibition depletes inositol and prevents the formation of IP_3 (Kennedy *et al*, 1990; Phiel and Klein, 2001). Animals pretreated with LiCl showed an impaired antinociceptive response to the administration of physostigmine and oxotremorine. The role of IP_3 in muscarinic analgesia was also confirmed by the dose-dependent prevention, induced by heparin, of the increase of pain threshold produced by cholinomimetic drugs. Furthermore, a shift to the right of the dose–response curve of physostigmine was produced by pretreatment with low molecular weight heparin. Heparin is a potent and selective IP_3 receptor antagonist (Jonas *et al*, 1997). This compound must be injected into cells or perfused onto permeabilized cells because of its high molecular weight

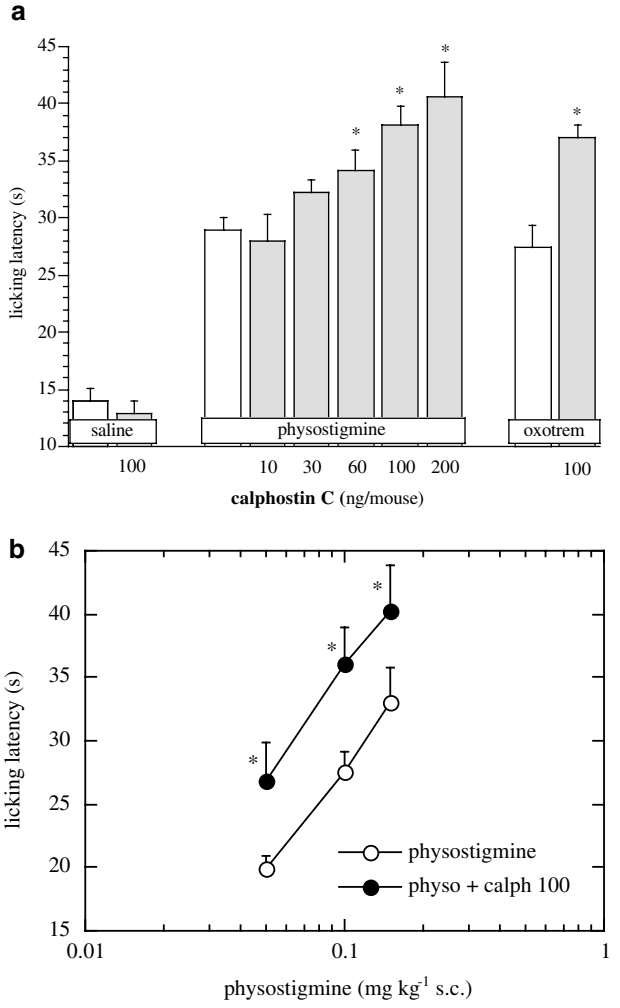

Figure 6 Effect of calphostin C on physostigmine and oxotremorine antinociception in the mouse hot-plate test. (a) Potentiation by calphostin C (10–200 ng per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice. (b) Shift to the left of the dose–response curve of physostigmine (0.05–0.15 mg kg^{-1} s.c.) by calphostin C (100 ng per mouse i.c.v.). Vertical lines represent SEM. *$P<0.05$ in comparison with physostigmine-treated mice.

(12 000–13 000 Da) and lack of membrane permeability. Some evidence indicates that the low molecular weight heparin (6000 Da) used in this study is membrane permeable. Perfusion of low molecular weight heparin over a nonpermeabilized cerebellar slice preparation attenuated glutamate-stimulated increases in free intracellular Ca^{2+} (Jonas *et al*, 1997). IP_3, through the interaction with specific receptors located on the endoplasmic reticulum, causes release of Ca^{2+} from intracellular stores into the cytoplasm (Mignery and Sudhof, 1990; Ferris *et al*, 1992). Since the present results indicate the importance of IP_3 production in the induction of muscarinic analgesia, we thought it worthwhile to investigate the role played by the variation of the intracellular Ca^{2+} levels in the mechanism of action of physostigmine and oxotremorine. To this purpose, TMB-8, an agent that antagonizes the mobilization of Ca^{2+} from intracellular stores (Malagodi and Chiou, 1974), was used. Pretreatment with TMB-8 dose-dependently antagonized the increase of pain threshold induced by cholinomimetics and shifted to the right the dose–response curve of physostigmine. The antagonism exerted by TMB-8 was completely reverted by coadministration of Ca^{2+}, suggesting that the release of Ca^{2+} from intracellular stores induced by IP_3 is necessary to produce muscarinic analgesia. To further support this hypothesis, it should be noted that also the prevention of muscarinic antinociception produced by inhibition of IP_3 synthesis with LiCl, as well as by antagonism of IP_3 receptor with heparin, was dose-dependently reverted by coadministration of Ca^{2+}. The i.c.v. administration of Ca^{2+} produced neither alteration of animals' gross nor induction of behavioral side effects, as previously observed for the same range of doses used in the present study (Vocci *et al*, 1980).

In addition to inducing IP_3 formation, PLC causes the activation of PKC through stimulation of the production of DAG (Rebecchi and Pentyala, 2000). Pretreatment with calphostin C, a very selective, potent, and membrane-permeable PKC inhibitor (Kobayashi *et al*, 1989), enhanced the antinociception induced by both physostigmine and oxotremorine. The administration of the PKC inhibitor also shifted to the left the dose–response curve of physostigmine. Furthermore, activation of PKC by phorbol esters, such as PMA and PDBu (Nishizuka, 1992), dose-dependently prevented the physostigmine and oxotremorine increase of pain threshold. These data clearly indicate that activation of PKC by cholinomimetics constitutes a significant pathway involved in negative modulation of central muscarinic antinociceptive response. We, therefore, propose that the intracellular negative feedback action by PKC on the central muscarinic antinociceptive pathway is necessary to maintain the basal level of pain sensitivity.

The highest active doses of U-73122, LiCl, heparin, TMB-8, PMA, and PDBu, as well as the inhibition of the expression of PLCβ_1, G_q, and G_{11} by means of selective aODNs, in the absence of coadministration of analgesic drugs, did not reduce the licking latency values of mice in comparison with control groups. Similarly, calphostin C that potentiated muscarinic analgesia, and Ca^{2+} that reverted the antagonism induced by TMB-8, LiCl and heparin, when injected alone, did not increase the mouse pain threshold. These results exclude not only that the prevention of physostigmine and oxotremorine antinociception is because of hyperalgesic effect of the intracellular modulators used, but also that the action produced by calphostin C and Ca^{2+} on muscarinic analgesia origins from antinociceptive properties of the compounds employed.

The receptor-mediated activation of the muscarinic system, as well as the modulation of the intracellular events promoted by cholinomimetics, can induce several side effects. It is widely known that physostigmine and oxotremorine can produce the typical cholinergic symptomatology (tremors, sialorrhea, diarrhea, lacrimation, etc). The PKC activator PMA and PDBu are convulsant (Smith and Meldrum, 1992) and, similarly, LiCl can induce neurological toxicity characterized by tremors, convulsion, ataxia (Kores and Lader, 1997). All the compounds, at the highest active doses employed in the present study, did not cause any detectable modification in mouse gross behavior. At the same doses, all treatments did not impair motor coordination nor modify spontaneous motility nor inspec-

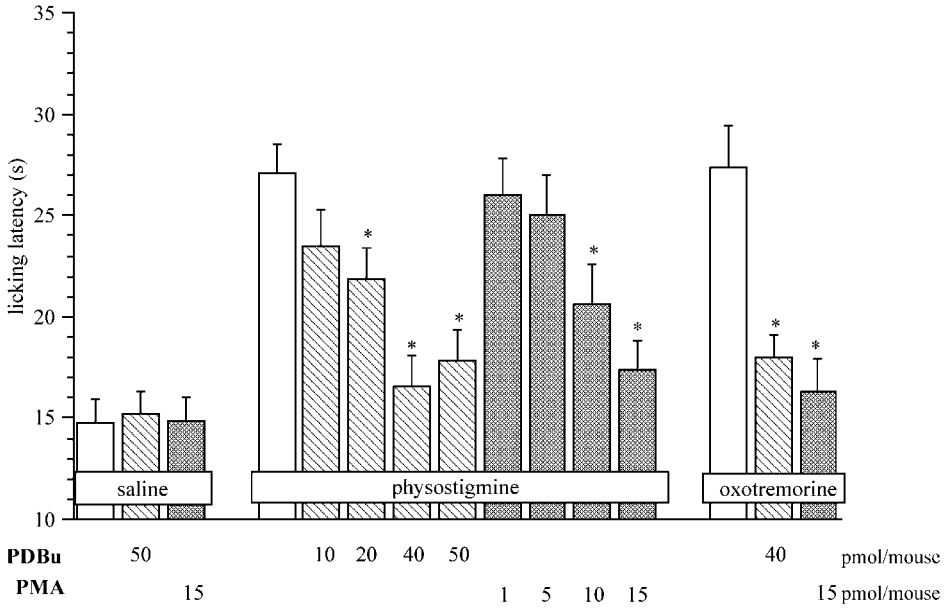

Figure 7 Prevention by pretreatment with PDBu (10–50 pmol per mouse i.c.v.) and by PMA (1–15 pmol per mouse i.c.v.) of physostigmine (0.1 mg kg^{-1} s.c.)- and oxotremorine (60 μg per mouse i.c.v.)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after cholinomimetics administration. Vertical lines represent SEM. *$P<0.05$ in comparison with corresponding analgesic drug-treated mice.

Table 1 Lack of Effect of Pretreatment with LiCl, TMB-8, Ca^{2+}, Heparin, U-73122, Calphostin C, PMA, PDBu, Anti-$G_{q\alpha}$, Anti-$G_{11\alpha}$, Anti-PLCβ_1 on Exploratory Activity and Spontaneous Motility in the Mouse Hole-Board Test

Treatment	Exploratory activity Counts in 5 min	Spontaneous motility Counts in 5 min
Saline	34.3 ± 2.6	56.9 ± 3.8
LiCl 500 mg/kg	40.4 ± 4.1	57.8 ± 1.7
TMB-8 1 μg/mouse	34.6 ± 6.6	57.8 ± 6.7
Ca^{2+} 0.1 μmol/mouse	32.5 ± 3.6	57.8 ± 1.7
Heparin 40 μg/mouse	33.9 ± 5.9	61.9 ± 6.1
Vehicle	38.2 ± 3.1	50.1 ± 5.1
U-73122 5 μg/mouse	38.8 ± 4.0	57.1 ± 5.1
Calphostin C 100 ng/mouse	37.8 ± 4.0	57.1 ± 5.0
PMA 15 pmol/mouse	38.3 ± 7.1	69.5 ± 13.0
PDBu 40 pmol/mouse	40.8 ± 7.2	54.4 ± 6.9
dODN 3 nmol/mouse	36.7 ± 4.0	56.3 ± 4.2
Anti-$G_{q\alpha}$ 3 nmol/mouse	34.8 ± 4.1	62.1 ± 3.6
Anti-$G_{11\alpha}$ 3 nmol/mouse	33.3 ± 4.7	53.8 ± 6.1
Anti-PLCβ_1 3 nmol/mouse	34.8 ± 1.6	56.2 ± 4.9
Amphetamine 2 mg/kg	58.2 ± 5.1*	104.8 ± 10.0*

*$P<0.05$ in comparison with saline-treated mice.

tion activity in comparison with control groups, excluding that the results obtained were because of animals' altered viability. It should be noted that higher doses of both cholinomimetics and intracellular modulators could not be investigated since they induced toxicity (tremors, convulsions, etc) in animals. The induction of toxicity can also be considered as an indication not only of the diffusion of these compounds in the brain, but also of the consequent reaching of key targets by using the administration schedule employed in the present study.

Seen as a whole, our data evidence the role of PLC-IP_3 pathway in the induction of cholinergic analgesia in mice. Furthermore, the concomitant activation of PKC through DAG generation induced by cholinomimetics can represent an autoinhibitory pathway involved in negative modulation of muscarinic antinociception.

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REFERENCES

Ashkenazi A, Winslow JW, Peralta EG, Peterson GL, Schimerlik MI, Capon DJ *et al* (1987). An m2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* **238**: 672–675.

Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A (1992). Role of muscarinic receptor subtypes in central antinociception. *Br J Pharmacol* **105**: 77–82.

Caulfield MP (1993). Muscarinic receptors—characterization, coupling and function. *Pharmacol Ther* **58**: 319–379.

Caulfield MP, Birdsall NJM (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50**: 279–290.

Felder CC (1995). Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J* **9**: 619–625.

Ferris CD, Cameron AM, Huganir RL, Snyder SH (1992). Quantal calcium release by purified reconstituted inositol 1,4,5-trisphosphate receptors. *Nature* **35**: 350–352.

Ghelardini C, Galeotti N, Bartolini A (2000). Loss of muscarinic antinociception by antisense inhibition of M_1 receptors. *Br J Pharmacol* **129**: 1633–1640.

Ghelardini C, Galeotti N, Gualtieri F, Romanelli MN, Bartolini A (1996). S-(−)-ET126: a potent and selective M_1 antagonist *in vivo* and *in vitro*. *Life Sci* **58**: 991–1000.

Gillbert PG, Gordh Jr T, Hartvig P, Jansson I, Petterson J, Post C (1989). Characterization of the antinociception induced by intrathecally administered carbachol. *Pharmacol Toxicol* **64**: 340–343.

Gomeza J, Zhang L, Kostenis E, Felder CC, Bymaster FP, Brodkin J *et al* (2001). Generation and pharmacological analysis

of M_2 and M_4 muscarinic receptor knockout mice. *Life Sci* **68**: 2457–2466.

Haley TJ, Mccormick WG (1957). Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol Chemother* **12**: 12–15.

Hartvig P, Gillberg PG, Gordh T, Post C (1989). Cholinergic mechanisms in pain and analgesia. *Trends Pharmacol Sci* **1**(Suppl.): 75–79.

Iwamoto ET, Marion L (1993). Characterization of the antinociception produced by intrathecally administered muscarinic agonists in rats. *J Pharmacol Exp Ther* **266**: 329–338.

Jonas S, Sugimori M, Llinas R (1997). Low molecular weight heparin a neuroprotectant? *Ann NY Acad Sci* **825**: 389–393.

Kennedy ED, Xhalliss RA, Rangan CI, Nahorski SR (1990). Reduced inositol polyphosphate accumulation and inositol supply induced by lithium in stimulated cerebral cortex slices. *Biochem J* **267**: 781–786.

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159**: 548–553.

Kores B, Lader MH (1997). Irreversible lithium neurotoxicity: an overview. *Clin Neuropharmacol* **20**: 283–299.

Malagodi MH, Chiou CY (1974). Pharmacological evaluation of a new Ca^{++} antagonist, 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studied in smooth muscle. *Eur J Pharmacol* **27**: 25–33.

Mignery GA, Sudhof TC (1990). The ligand binding sites and transduction mechanism in the inositol-1,4,5-triphosphate receptor. *EMBO J* **9**: 3893–3898.

Naguib M, Yaksh TL (1997). Characterization of muscarinic receptor subtypes that mediate antinociception in the rat spinal cord. *Anesth Analg* **85**: 847–853.

Nishizuka Y (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**: 607–614.

O'Callaghan JP, Holtzman SG (1975). Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *J Pharmacol Exp Ther* **192**: 497–505.

Pellandra CL (1933). La geneserine-morphine adjuvant de l'anesthesia generale. *Lyon Med* **151**: 653.

Phiel CJ, Klein PS (2001). Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol* **41**: 789–813.

Rebecchi MJ, Pentyala SN (2000). Structure, function and control of phosphoinositide-specific phospholipase C. *Physiol Rev* **80**: 1291–1335.

Sanchez-Blazquez P, Garzon J (1998). Delta opioid subtypes activates inositol-signaling pathways in the production of antinociception. *J Pharmacol Exp Ther* **285**: 820–827.

Smith SE, Meldrum BS (1992). The protein kinase C activators, phorbol 12-myrystate,13-acetate and phorbol 12,13-dibutyrate, are convulsant in the pico-nanomolar range in mice. *Eur J Pharmacol* **213**: 133–135.

Taylor SJ, Chae HZ, Rhee SG, Exton JH (1991). Activation of β1 isozyme of phospholipase C by α subunits of the Gq class of G protein. *Nature* **350**: 516–518.

Vaught J, Pelley K, Costa LG, Sether P, Enna SJ (1985). A comparison of the antinociceptive responses to GABA-receptor agonists THIP and baclofen. *Neuropharmacology* **24**: 211–216.

Vocci FJ, Welch SP, Dewey WL (1980). Differential effects of divalent cations, cation chelators and ionophore (A23187) on morphine and dibutyryl guanosine 3′:5′-cyclic monophosphate antinociception. *J Pharmacol Exp Ther* **214**: 463–466.

Wakdo GL, Northup J, Perkins JP, Harden TK (1983). Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. *J Biol Chem* **258**: 13900–13908.

Yule DI, Williams JA (1992). U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J Biol Chem* **267**: 13830–13835.