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Mechanisms involved in the antinociceptive effect caused by diphenyl diselenide in the formalin test

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Abstract

This study investigated the mechanisms involved in the antinociceptive action induced by diphenyl diselenide ($(PhSe)_2$) in the formalin test. Mice were pre-treated with $(PhSe)_2$ by the oral route (0.1–100 mg kg^{-1}), 30 min before formalin injection. To address some of the mechanisms by which $(PhSe)_2$ inhibits formalin-induced nociception mice were treated with different drugs. The antinociceptive effect of $(PhSe)_2$ was shown in the first and second phases of the formalin test. The antinociceptive effect caused by $(PhSe)_2$ (10 mg kg^{-1}, p.o.) was prevented by intrathecal injection of K^+ channel blockers such as apamin and charybdotoxin (small- and large-conductance Ca^{2+}-activated K^+ channel inhibitors, respectively) and tetraethylammonium (TEA, a non-selective voltage-dependent K^+ channel inhibitor), but not glibenclamide (an ATP-sensitive K^+ channel inhibitor). The antinociceptive action caused by $(PhSe)_2$ (10 mg kg^{-1}, p.o.) was also blocked by a nitric oxide (NO) synthase inhibitor (N^{ω}-nitro-L-arginine, L-NOARG) and the soluble guanylate cyclase inhibitors 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) and methylene blue. These results suggest the participation of NO/cyclic GMP/Ca^{2+} and K^+ channel pathways in the antinociceptive effect caused by $(PhSe)_2$.

Introduction

Selenium compounds display antioxidant, neuroprotective, antihypertensive, anticancer, antiviral, immunosuppressive, antimicrobial and anti-inflammatory properties (Sies 1993; Schewe 1995; May 1999; Nogueira et al 2003; Meotti et al 2004; Zasso et al 2005; Savegnago et al 2007a, b, c). Because of this, a great number of novel pharmaceutical agents that are selenium-based or that target specific aspects of selenium metabolism are under development (May 1999; Nogueira et al 2003; Meotti et al 2004).

The organic selenium compound, ebselen, is a classical antioxidant and well known as the most important glutathione peroxidase mimetic agent (Daiber et al 2000). Further to its peroxidase-like activity, ebselen has anti-inflammatory activity in different models of inflammation (Parnham & Graf 1987; Schewe 1995), which may be related at least in part to its capability of scavenging peroxynitrite, a potent inflammatory mediator (Sies & Arteel 2000). The mechanism(s) underlying the anti-inflammatory activity of ebselen is still not completely understood but is linked to inhibition of NADPH-oxidase, protein kinase C, nitric oxide synthase and lipoxygenases, most likely by interacting with critical thiol/disulfide groups in these enzymes (Cotgreave et al 1989; Walther et al 1999; Mugesh et al 2001).

Similarly, diphenyl diselenide, $(PhSe)_2$, a simple diaryl diselenide, is known to induce minor toxic effects when administered acutely to mice at doses that have antinociceptive and anti-inflammatory activity (Nogueira et al 2003; Zasso et al 2005; Savegnago et al 2007a). We have previously reported that $(PhSe)_2$ causes an antinociceptive effect in several models of pain through a complex mechanism that likely involves an interaction with the serotoninergic, glutamatergic and nitrergic pathways, as well as peptidergic and vanilloid systems (Savegnago et al 2007a, b). More recently, we demonstrated that $(PhSe)_2$ produces a systemic anti-allodynic action in two models of persistent inflammation and neuropathic pain and attenuates acute hyperalgesia induced by glutamate, NMDA, bradykinin and PGE_2 in mice (Savegnago et al 2007c).

Nitric oxide (NO) is an important messenger in the central nervous system. It has been suggested that NO may be involved in the pathophysiology of Alzheimer's disease, cerebral ischaemia, alcohol-induced brain damage, long-term potentiation, learning, memory, wakefulness, circadian rhythm, nociception, olfaction, food intake, drinking, regulation of

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neurotransmitter release and anxiety (Dawson & Dawson 1996). Some reports have indicated that NO affects many functions, such as pain (Souza & Prado 2001; Prado et al 2002), synaptic plasticity (Li & Wieranszko 1994) and neuronal damage (Li & Wieranszko 1994; Nara et al 1999) biphasically. In agreement, there is evidence that the L-arginine/NO/cGMP pathway may be involved in peripheral and central nociceptive processing (Duarte et al 1990; Souza & Prado 2001). It has been reported that increased NO production induces hyperalgesia and that NO synthase (NOS) inhibitors can suppress pain. Paradoxically, results have also implicated NO as a mediator or modulator in analgesic drug function (Souza & Prado 2001).

Many studies have indicated that K^+ channels play a key role in setting the resting membrane potential and in controlling neuronal activity and signal propagation throughout the nervous system (Hille 2001). Others have focused on the role of K^+ channels in pain. It has been reported that these channels contribute to the modulation of pain perception in the central nervous system (Ocano et al 2004). While administration of K^+ channel openers (i.c.v.) results in antinociception, application of K^+ channel blockers reduces antinociception produced by morphine (Ocano et al 1990).

Nowadays, few drugs are effective in treating pathological (acute and chronic) pain and, in general, these drugs present low efficacy and numerous side effects (Woolf & Mannion 1999; Mendell & Sahenk 2003). For that reason, the search for new compounds that could be applied in acute and chronic pain therapy has been essential.

Thus, despite the growing amount of experimental data, the precise mechanisms through which $(PhSe)_2$ causes systemic antinociception in rodents still remain elusive. Therefore, the goal of this study was to further investigate the mechanisms involved in the antinociceptive action caused by $(PhSe)_2$. To this end, we carried out experiments to determine the possible participation of the NO/cGMP/Ca^{2+} and K^+- channel pathways in the antinociceptive effect induced by $(PhSe)_2$ on the formalin test.

Materials and Methods

Drugs

Diphenyl diselenide $(PhSe)_2$ was prepared and characterized in our laboratory by the method previously described (Paulmier 1986). Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of $(PhSe)_2$ (99.9%) was determined by using GC/FID and GC/MS. $(PhSe)_2$ was dissolved in canola oil. All other chemicals were of analytical grade and obtained from standard commercial suppliers. All drugs were dissolved in saline, with the exception of ODQ and glibenclamide, which were dissolved in 10% dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.5% and had no detectable effect per se when assessed in control mice.

Animals

The behavioural experiments were conducted using male Swiss mice, 25–35 g, from our own breeding colony. The mice were kept in separate animal rooms, on a 12-h light–dark cycle (lights on at 0600 h), at air-temperature (23–25°C), with free access to food and water. Mice were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experimenters were blinded to the drugs given to mice. In each experimental condition, the number of mice used was 7–10. At the end of the experiments, mice were euthanized by cervical displacement.

The mice were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann 1983). The number of mice and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Intracerebroventricular and intrathecal injections

For intracerebroventricular injections, mice were lightly anaesthetized and a volume of 5 μL of sterile saline or the drugs were directly injected into the lateral ventricle (coordinates from bregma: 1 mm lateral, 1 mm rostral, 3 mm vertical) as described previously (Vaz et al 1996).

The intrathecal injections were made in conscious mice using the method described by Hylden & Wilcox (1980). The mice received, using a microsyringe connected to polyethylene tubing, a volume of 5 μL of sterile saline (control) or drugs injected directly between the subdural space of the L5–L6 spinal segments. Injections were given over a period of 5 s (Vaz et al 1996). The injection site was verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of injected dye in the spinal cord. The success rate for the injections was consistently found to be 95% before the experiments were done.

Effect of diphenyl diselenide on formalin-induced nociception and oedema

The formalin test was carried out as described by Hunskaar & Hole (1987). Mice received 20 μL of 2.5% formalin solution (0.92% of formaldehyde), injected intraplantarly in the ventral right hindpaw. Mice were pre-treated with $(PhSe)_2$ by the oral route (0.1–100 mg kg^{-1}; 10 mL kg^{-1}), 30 min before formalin injection. This time was chosen based on a time-course study (Savegnago et al 2007a). Control animals received a similar volume of vehicle (canola oil; 10 ml/kg; p.o.). After intraplantar injection of formalin, the time spent licking the injected paw was recorded with a chronometer during the periods of 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) and considered as indicative of nociception.

To assess whether the antinociceptive activity produced by $(PhSe)_2$ in formalin-induced nociception was associated with development of anti-oedematogenic activity, we measured paw oedema by comparing the difference between the weight of the formalin-treated paw and the weight of the contralateral paw (non-treated paw). For this purpose, mice were euthanized 30 min after formalin injection by cervical dislocation, and both paws were cut at the ankle joint and weighed on an analytical

balance. This procedure was similar to that described previously (Beirith et al 1998).

In a separate set of experiments, we investigated the antinociceptive effect of $(PhSe)_2$ given after formalin injection. The challenge test was evaluated only in the second phase of the formalin test, 15–30 min after formalin injection. For this purpose, mice were intraplantarly injected with formalin and 10 (post-administered) or 30 min (pre-administered) before the second phase they received $(PhSe)_2$ (10 mg kg^{-1}, p.o.) or vehicle (canola oil, p.o.) for evaluation against the second phase of the nociception induced by formalin. The interval between administrations of $(PhSe)_2$ and the second phase of the experiment is different. The difference of administration timing was necessary to determine whether $(PhSe)_2$ has therapeutic (post-administered) or prophylactic effects (pre-administered).

Analysis of the possible mechanism of action of diphenyl diselenide in the formalin test

To address some of the mechanisms by which $(PhSe)_2$ inhibits formalin-induced nociception, mice were treated with different drugs. The choice of the doses of each drug was based on previous data from the literature. The formalin model was chosen for this purpose because of the specificity and sensitivity in nociception transmission that this model provides (Le Bars et al 2001).

Involvement of nitric oxide/cyclic GMP pathway in the antinociceptive action caused by diphenyl diselenide in the formalin test

The mice were pre-treated with L-NOARG (an NO synthase inhibitor; 456 nmol/site, i.c.v.), ODQ (a soluble guanylate cyclase inhibitor; 0.3 nmol/site, i.c.v.) and methylene blue (an inhibitor of NO synthase and soluble guanylate cyclase; 1 mg kg^{-1}, i.p.) 10 min before $(PhSe)_2$ (10 mg kg^{-1}, p.o.) administration. Other groups of mice received only $(PhSe)_2$ (10 mg kg^{-1}, p.o.) or vehicle (canola oil, 10 mL kg^{-1}, p.o., or sterile saline 5 μL/site; i.c.v.) 30 min before formalin injection. The nociceptive response in the first and second phases of the formalin test was recorded 30 min after administration of $(PhSe)_2$ or vehicle. The dose of methylene blue was based on the work of Jain et al (2001) and the doses of L-NOARG and ODQ were based on Ferreira et al (1999).

Possible involvement of K^+ channels in the antinociceptive effect of diphenyl diselenide in the formalin test

For this purpose, mice were pre-treated with glibenclamide (100 (g/site, i.t.; a blocker of ATP-sensitive K^+ channels), apamin (50 ng/site, i.t.; a blocker of small (or low)-conductance Ca^{2+}-sensitive K^+ channels), charybdotoxin (250 pg/site, i.t.; a blocker of large (or fast)-conductance Ca^{2+}-sensitive K^+ channels) or tetraethylammonium (TEA; 1 μg/site, i.t.; a non selective blocker of voltage-sensitive K^+ channels), and after 15 min they received $(PhSe)_2$ (10 mg kg^{-1}, p.o.) or morphine (2.5 mg kg^{-1}, s.c.), used as a positive control, or vehicle. Other groups of mice received saline (5 μL/site) by the intrathecal route, 15 min before the administration of $(PhSe)_2$, morphine or vehicle. The nociceptive responses to formalin were recorded 30 or 20 min after the administration of $(PhSe)_2$ or morphine, respectively. The procedure and doses used were essentially similar to those described previously (Strong 1990; Aronson 1992; Welch & Dunlow 1993; Santos et al 1999).

Statistical analysis

The results are presented as the mean ± s.e.m., except the ID50 values (i.e., the dose of $(PhSe)_2$ reducing the pain response by 50% in relation to control group values). The statistical significance between groups was assessed by means of one-way analysis of variance followed by Student–Newman–Keuls test when appropriate. $P < 0.05$ was considered to be indicative of significance. The ID50 values were determined by linear regression analysis from individual experiments using GraphPad Software 4.0 (GraphPad, USA). The percentages of inhibition were calculated for the maximal developed responses in comparison with vehicle-treated mice.

Results

Effect of diphenyl diselenide on formalin-induced nociception

The results in Figure 1A show that $(PhSe)_2$ (10–100 mg kg^{-1}, p.o.) caused a significant inhibition in the first phase (0–5 min) of the formalin-induced licking. Furthermore, $(PhSe)_2$ administered orally at doses of 1–100 mg kg^{-1} elicited significant inhibition in the second (15–30 min) phase of the formalin test (Figure 1B). The calculated mean ID50 for these effects were: 25.55 (9.52–68.58) mg kg^{-1} for the first phase and 6.45 (1.75–23.8) mg kg^{-1} for the second phase, and the percentages of inhibition observed were 76 ± 3 and 97 ± 1%, respectively.

$(PhSe)_2$ (0.1–100 mg kg^{-1}, p.o.) was significantly effective in inhibiting the mouse paw oedema induced by intraplantar injection of formalin (percentage of inhibition of 43 ± 4%) (Figure 1C).

The oral administration of $(PhSe)_2$ (10 mg kg^{-1}, given 10 min before the second phase, post-administered $(PhSe)_2$) in mice, caused a significant inhibition (71 ± 6%) in the second phase (inflammatory nociception) of the formalin test. The prophylactic treatment with $(PhSe)_2$ (10 mg kg^{-1}, p.o.), given 30 min before the second phase in mice, produced a significant inhibition (89 ± 3%) of the nociception in the second phase of the formalin test (Figure 1D).

Antagonism of diphenyl diselenide-induced antinociception by L-NOARG, ODQ and methylene blue in the formalin test

The intracerebroventricular administration of an inhibitor of NO synthase, L-NOARG (456 nmol/site), significantly reduced the antinociceptive action induced by $(PhSe)_2$ (10 mg kg^{-1}, p.o.) in the first (0–5 min) and in the second (15–30 min) phases of the formalin test (Table 1).

The intracerebroventricular injection of an inhibitor of guanylate cyclase, ODQ (0.3 nmol/site), significantly inhibited

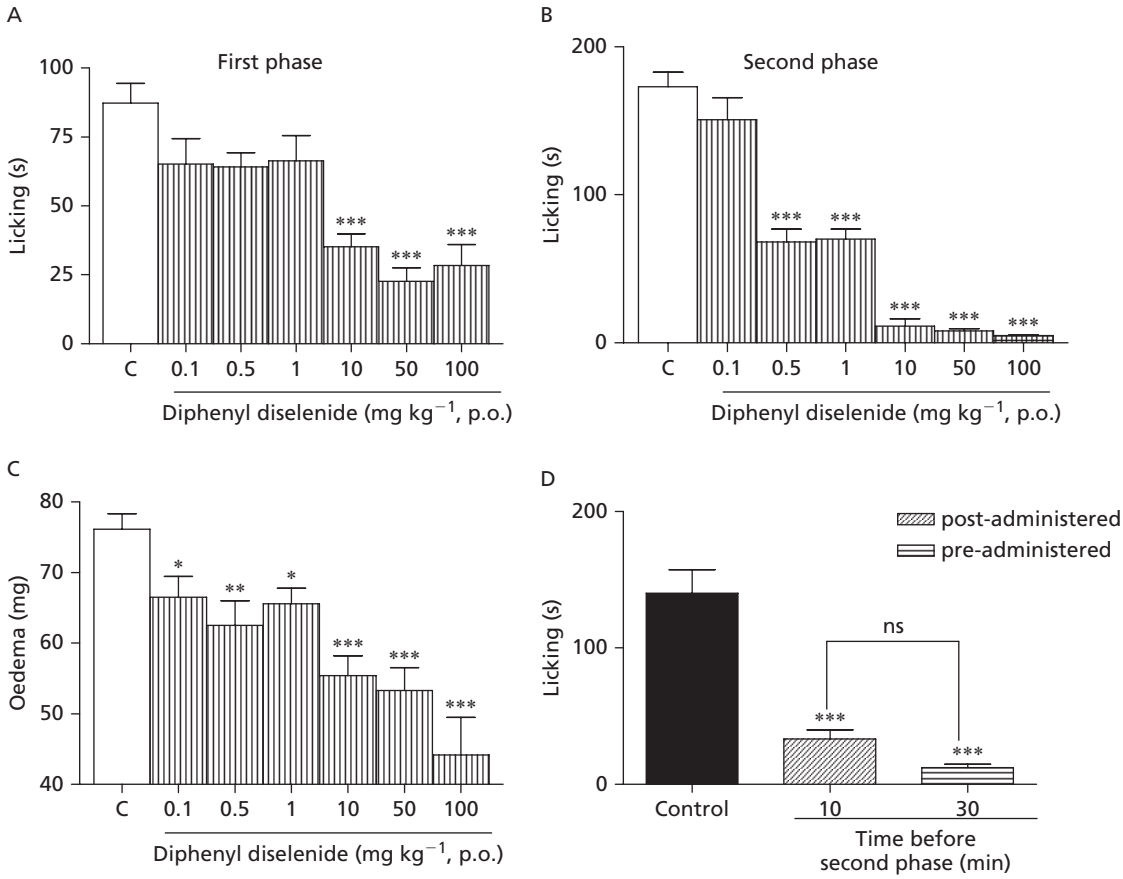

Figure 1 Effect of diphenyl diselenide given by the oral route (0.1–100 mg kg^{-1}, p.o.) on the licking (A, B) and oedema (C) induced by formalin in mice. The total time spent licking the hindpaw was measured in the first (0–5 min, panel A) and the second (15–30 min, panel B) phases after intraplantar injection of formalin. The oedema (panel C) was measured at the end of the second phase of the formalin test. Mice were intraplantarly injected with formalin, and 10 (post-treatment) or 30 (pre-treatment) min before the second phase they received $(PhSe)_2$ (10 mg kg^{-1}, p.o.) or vehicle (canola oil, p.o.) for evaluation against the second phase of the nociception induced by formalin (panel D). Total time spent licking the hindpaw was measured in the second phase (15–30 min) of the formalin test (panel D). For more details see Material and Methods section. The control group mice were injected with vehicle (canola oil). Each column represents the mean of 7–10 mice and vertical lines indicate the s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control group (one-way analysis of variance followed by Newman–Keuls test); ns, denotes no significant difference between mouse pre-administered and post-administered with $(PhSe)_2$.

the $(PhSe)_2$-induced antinociceptive effect in the second (but not in the first) phase of the formalin test. Methylene blue (1 mg kg^{-1}, i.p.) significantly attenuated the $(PhSe)_2$-induced antinociceptive effect in the second phase of the formalin test (Table 1).

Involvement of K^+ channels in the antinociceptive action caused by diphenyl diselenide in the formalin test

Intrathecal administration of TEA (1 μg/site), charybdotoxin (250 pg/site) and apamin (50 ng/site), given 15 min beforehand, prevented the antinociception caused by $(PhSe)_2$ (10 mg kg^{-1}) in the second (but not in the first) phase of the formalin test (Table 2).

Conversely, glibenclamide (100 μg/site) failed to affect the antinociceptive action of $(PhSe)_2$ (10 mg kg^{-1}) against either phase of the formalin test. Treatment with apamin, charybdotoxin, TEA or glibenclamide significantly reversed the antinociceptive effect of morphine when assessed against the first and second phases of the formalin test (Table 2).

Discussion

The results of this study demonstrated that $(PhSe)_2$ administered orally elicited a significant antinociception action in mice, when assessed in the formalin model. In fact, this compound produced graded inhibition against neurogenic (first phase, 0–5 min) (76 ± 3%) and inflammatory (second phase, 15–30 min) (97 ± 1%) pain responses caused by formalin injection in mice. Indeed, $(PhSe)_2$ was also effective in inhibiting (43 ± 4%) the mouse paw oedema induced by intraplantar injection of formalin. Thus, these results suggest that $(PhSe)_2$ was effective in reducing inflammatory pain on the formalin test and demonstrated that antinociception caused by $(PhSe)_2$ seems to be associated with its anti-inflammatory action, as revealed by inhibition of paw oedema formation in the mice treated with formalin. The

Table 1 Effect of pretreatment of mice with L-NOARG, ODQ and methylene blue before injection of diphenyl diselenide $(PhSe)_2$ against the first and the second phases of formalin-induced licking

Group	First phase	Second phase
Vehicle	87.40 ± 7.05	144.90 ± 11.88
L-NOARG	89.10 ± 9.43	138.6 ± 8.97
$(PhSe)_2$	23.10 ± 6.34***	19.38 ± 3.0***
L-NOARG/$(PhSe)_2$	73.56 ± 9.17#	111.10 ± 9.11#
Vehicle	88.82 ± 10.12	164.60 ± 7.23
ODQ	87.71 ± 7.12	179.0 ± 10.89
$(PhSe)_2$	20.03 ± 6.34***	15.70 ± 4.07***
ODQ/$(PhSe)_2$	69.80 ± 3.57#	153.41 ± 14.15#
Vehicle	93.55 ± 6.92	160.25 ± 11.62
Methylene blue	84.40 ± 5.03	155.84 ± 11.20
$(PhSe)_2$	26.65 ± 4.11***	17.63 ± 3.02**
Methylene blue/$(PhSe)_2$	42.50 ± 3.61***	61.20 ± 11.02#

Pre-treatment of mice was with L-NOARG (456 nmol/site, i.c.v.), ODQ (0.3 nmol/site, i.c.v.) and methylene blue (1 mg kg^{-1}, i.p.) 10 min before injection of diphenyl diselenide (10 mg kg^{-1}, p.o.) against the formalin-induced licking in mice. The total time spent licking the hindpaw was measured in the first (0–5 min) and in the second phases (15–30 min) after intraplantar injection of formalin. Values are expressed as mean ± s.e.m. (n = 5–7 mice/group). ***$P < 0.001$, compared with the vehicle group; **$P < 0.01$, compared with the vehicle group; #$P < 0.05$, compared with the diphenyl diselenide group (one-way analysis of variance followed by Newman–Keuls test).

Table 2 Effect of pretreatment of mice with TEA, charybdotoxin, apamin and glibenclamide before injection of diphenyl diselenide $(PhSe)_2$ against the first and the second phases of formalin-induced licking in mice

Group	First phase	Second phase
Vehicle	110.31 ± 8.34	215.91 ± 13.50
TEA	103.22 ± 11.22	195.64 ± 12.92
Morphine	3.60 ± 1.63***	3.54 ± 0.92***
TEA/morphine	27.02 ± 3.29#	25.67 ± 4.29#
$(PhSe)_2$	53.77 ± 5.96***	26.40 ± 6.99***
TEA/$(PhSe)_2$	49.40 ± 4.73***	123.05 ± 16.14#
Vehicle	115.31 ± 10.72	205.97 ± 10.71
Charybdotoxin	92.17 ± 12.58	190.77 ± 15.13
Morphine	4.71 ± 1.25***	5.02 ± 0.97***
Charybdotoxin/morphine	35.60 ± 3.87#	43.25 ± 6.39#
$(PhSe)_2$	54.07 ± 7.67***	30.17 ± 6.08***
Charybdotoxin/$(PhSe)_2$	53.30 ± 6.18***	218.8 ± 35.57#
Vehicle	105.44 ± 8.71	198.75 ± 12.71
Apamin	97.60 ± 4.80	182.61 ± 5.97
Morphine	5.32 ± 1.77***	6.07 ± 1.22***
Apamin/morphine	30.60 ± 3.21#	78.20 ± 8.44#
$(PhSe)_2$	48.91 ± 4.22***	25.54 ± 3.07***
Apamin/$(PhSe)_2$	64.05 ± 7.28**	78.75 ± 4.27#
Vehicle	93.30 ± 10.77	172.02 ± 9.24
Glibenclamide	83.44 ± 9.56	167.05 ± 11.47
Morphine	4.01 ± 0.56***	4.51 ± 1.89***
Glibenclamide/morphine	90.07 ± 8.88#	87.55 ± 3.21#
$(PhSe)_2$	44.38 ± 4.23***	14.64 ± 2.23***
Glibenclamide/$(PhSe)_2$	37.39 ± 5.01***	21.24 ± 4.17***

Pre-treatment of mice with tetraethylammonium (TEA; 1 μg/site, i.t.), charybdotoxin (250 pg/site, i.t.), apamin (50 ng/site, i.t.) and glibenclamide (100 μg/site, i.t.) in the antinociceptive profiles by diphenyl diselenide (10 mg kg^{-1}, p.o) and morphine (2.5 mg kg^{-1}) against formalin-induced nociception in mice. The total time spent licking the hindpaw was measured in the first (0–5 min) and the second phases (15–30 min) after intraplantar injection of formalin. Values are expressed as mean (s.e.m. (n = 5–7 mice/group). **$P < 0.01$, ***$P < 0.001$, compared with the vehicle group; #$P < 0.05$, compared with diphenyl diselenide or morphine alone (one-way analysis of variance followed by Newman–Keuls test).

reduction in pain sensitivity of the inflammatory pain (second phase) and paw oedema caused by $(PhSe)_2$ could be related to inhibition of prostaglandin synthesis at the level of cyclooxygenases (COX). In agreement, it has been demonstrated that the nociceptive response caused by intraplantar injection of formalin, together with formation of oedema, is associated with release of several inflammatory mediators, including prostaglandins (Tjølsen et al 1992). These results are consistent with our early study in which $(PhSe)_2$, injected by the subcutaneous route, caused an antinociceptive action in the formalin test (Nogueira et al 2003). Moreover, our research group has reported that systemic (into the contralateral paw) or local (ipsilateral paw) administration of $(PhSe)_2$ reduced the pain-licking response in the second phase of the formalin test, thus confirming that it displays antinociceptive properties. The systemic effect of $(PhSe)_2$ suggests the involvement of a central action in the antinociceptive property of this organoselenium compound (Zasso et al 2005). The use of morphine in this study was due to the fact that morphine has antinociceptive effect with involvement of the NO/cGMP/K^+ channel pathway (Ocano et al 1990; Ferreira et al 1991). Since this study was carried out to determine the possible participation of the NO/cGMP/Ca^{2+} and K^+- channel pathways in the antinociceptive effect induced by $(PhSe)_2$ in the formalin test, we compared morphine with $(PhSe)_2$.

The formalin test in mice is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The noxious stimulus is an injection of dilute formalin under the skin of the surface of the right hindpaw. The response is the amount of time the mouse spends licking the injected paw. Two distinct periods of high licking activity can be identified, an early phase lasting for the first 5 min and a late phase lasting from 15 to 30 min after the injection of formalin. It is suggested that the early phase is due to a direct effect on nociceptors and that prostaglandins do not play an important role during this phase. The late phase seems to be an inflammatory response with inflammatory pain that can be inhibited by anti-inflammatory drugs (Hunskaar & Hole 1987; Tjølsen et al 1992). It has been demonstrated that intraplantar injection of formalin in rodents produces a significant increase in the spinal levels of different mediators, such as excitatory amino acids, prostaglandin E_2, substance P (SP), serotonin (5-HT) and histamine, among other peptides (Tjølsen et al 1992).

The results of our study demonstrate that $(PhSe)_2$, when examined in the formalin test, produced not only prophylactic (pre-administered) but also therapeutic effect (post-administered). Thus, these findings might have additional therapeutic implications for the development of a new drug to treat inflammatory pain. Another important objective of this study was to further characterize some of the mechanisms through which $(PhSe)_2$ exerts its antinociceptive action in the formalin test.

We reported here, for the first time, that Ca^{2+}-activated and non-selective voltage-dependent K^+ channel inhibitors caused a significant inhibition of the antinociception induced by systemic administration of $(PhSe)_2$ when analysed in the second phase of the formalin model of pain. However, the opening of ATP-sensitive K^+ channels does not appear to play a major role in $(PhSe)_2$-induced antinociception because treatment of mice with glibenclamide had no effect on $(PhSe)_2$ action when assessed against either phase of the formalin test. Our data are in agreement with those of other authors who have demonstrated that the peripheral antinociceptive effects produced by metamizole, meloxicam and resveratrol were blocked by the administration of Ca^{2+}-activated and non-selective voltage-dependent K^+ channel inhibitors, but not by the ATP-sensitive K^+ channel blocker, glibenclamide (Granados-Soto et al 2002; Ortiz et al 2003a, b, 2005). Interestingly, analysing the results obtained here it appears that the ion channel blockers affect the activity of morphine, a potent analgesic drug, much more than they do that of $(PhSe)_2$. Moreover, we can not discount the possibility that the observed effect of $(PhSe)_2$ could be due to an action on other K^+ channels. The fact that tetraethylammonium (TEA, a non-selective inhibitor of voltage-dependent K^+ channels) is also able to block Ca^{2+}-activated K^+ channels (Cook & Quast 1990) further suggests the participation of these channels in the mechanism of $(PhSe)_2$ action. The opening of K^+ channels, causing hyperpolarization of the cell membrane, is a physiological means of decreasing cell excitability. Thus, drugs with this property have a broad clinical potential. The identification of synthetic molecules that evoke physiological responses by opening K^+ channels has led to a new direction in the pharmacology of ion channels (Lawson 2000).

Also worthy of note was that the data provide pharmacological evidence for the involvement of the NO/cGMP pathway in the antinociceptive effect caused by $(PhSe)_2$ in the formalin test. This assertion is supported by the following evidence. First, the intracerebroventricular administration of L-NOARG (an NO synthase inhibitor) significantly reversed the antinociception caused by $(PhSe)_2$ in both phases of the formalin test. NO is generated from L-arginine by the catalytic action of NO synthase (NOS). Physiologically, the actions of NO are believed to be mediated by locally produced NO and, in most instances, by the subsequently generated second messenger molecule, guanosine 3′,5′-cyclic monophosphate (cGMP). The second piece of evidence is the fact that the pre-treatment of mice with guanylate cyclase inhibitors (methylene blue and ODQ) significantly reversed the antinociceptive effect induced by $(PhSe)_2$. Although methylene blue (inhibits both NOS and soluble guanylate cyclase (sGC)) is apparently not specific for soluble guanylate cyclase inhibition, it has been used as a pharmacological tool to inhibit this enzyme (Jain et al 2001; Patil et al 2004). Moreover, the data on ODQ reinforce the possible involvement of the NO/cGMP pathway in the antinociceptive action caused by $(PhSe)_2$, since ODQ is a highly NO-sensitive inhibitor of sGC.

Several other studies point to an antinociceptive effect via activation of the L-arginine/NO/cGMP pathway (Germany et al 1996). However, studies in the literature indicate that the NO/cGMP pathway can have pronociceptive, rather than antinociceptive, effects (Aley et al 1998). This discrepancy may be due to the different experimental pain models used, diverse tissue level and the variant NO and cGMP intracellular contents (Kawabata et al 1993; Mixcoatl-Zecuatl et al 2000; Tegeder et al 2002). Nevertheless, it is important to point out that in the rat formalin and the rat paw pressure models the production of NO and cGMP in subcutaneous tissue is involved in antinociceptive states (Soares et al 2000; Soares & Duarte 2001). It is very important to mention that in this study, the effect caused by $(PhSe)_2$ on the motor coordination of the mice was not evaluated. Zasso et al (2005) have reported that $(PhSe)_2$ causes no alteration on locomotion behaviour in the open field test, suggesting that this compound has no sedative effect (side effects).

Therefore, based on the results we can speculate that $(PhSe)_2$ could exert its antinociceptive effect due to the activation of K^+ channels at the spinal level, NO/cGMP at the supraspinal level and cGMP at the systemic level. Thus, collectively, these results suggest a major participation of the NO/cGMP pathway and Ca^{2+}/K^+ channels in the antinociceptive effect caused by $(PhSe)_2$ in the formalin test. Although its precise site of action remains unclear, these results suggest that $(PhSe)_2$ might be of potential interest in the development of new clinically relevant drugs for the management of pain.

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