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# Effect of diabetes on the mechanisms of intrathecal antinociception of sildenafil in rats

Claudia Ivonne Araiza-Saldaña [a], Gerardo Reyes-García [b], Deysi Yadira Bermúdez-Ocaña [a], Francisca Pérez-Severiano [c], Vinicio Granados-Soto [a,*]

[a] *Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados-Coapa, Calzada de los Tenorios 235, Colonia Granjas Coapa, 14330 México, D.F., Mexico*

[b] *Escuela Superior de Medicina del Instituto Politécnico Nacional, México, D.F., Mexico*

[c] *Departamento de Neuroquímica, Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez, México, D.F., Mexico*

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**Abstract**

The mechanism of intrathecal antinociceptive action of the phosphodiesterase 5 inhibitor sildenafil was assessed in diabetic rats using the formalin test. Intrathecal administration of sildenafil (12.5–50 μg) produced a dose-related antinociception during both phases of the formalin test in non-diabetic and diabetic rats. Intrathecal pretreatment with *N*-L-nitro-arginine methyl ester (L-NAME, nitric oxide (NO) synthase inhibitor, 1–50 μg), 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ, guanylyl cyclase inhibitor, 1–10 μg), KT5823 (protein kinase G (PKG) inhibitor, 5–500 ng), charybdotoxin (large-conductance $Ca^{2+}$-activated $K^+$ channel blocker, 0.01–1 ng), apamin (small-conductance $Ca^{2+}$-activated $K^+$ channel blocker, 0.1–3 ng) and glibenclamide (ATP-sensitive $K^+$ channel blocker, 12.5–50 μg), but not *N*-D-nitro-arginine methyl ester (D-NAME, 50 μg) or saline, significantly diminished sildenafil (50 μg)-induced antinociception in non-diabetic rats. Intrathecal administration of ODQ, KT5823, apamin and glibenclamide, but not L-NAME nor charybdotoxin, reversed intrathecal antinociception induced by sildenafil in diabetic rats. Results suggest that sildenafil produces its intrathecal antinociceptive effect via activation of NO–cyclic GMP–PKG–$K^+$ channels pathway in non-diabetic rats. Data suggest that diabetes leads to a dysfunction in NO and large-conductance $Ca^{2+}$-activated $K^+$ channels. Sildenafil could have a role in the pharmacotherapy of diabetes-associated pain.


*Keywords:* Sildenafil; Cyclic GMP; Protein kinase G; $K^+$ channel; Spinal processing

## 1. Introduction

Diabetes mellitus is one of the most common chronic medical conditions affecting over 100 million people worldwide, of whom up to 60% may develop diabetic neuropathy (Galer et al., 2000). The treatment of pain in diabetic patients is frequently unsatisfactory. Anticonvulsants, tricyclic antidepressants and opioids have become the mainstay in the treatment of chronic neuropathic pain (Sindrup and Jensen, 1999). However, these drugs often have a limited effect or they may cause intolerable side effects. Therefore, other options of treatment are needed. Sildenafil is an inhibitor of the cyclic GMP-specific phosphodiesterase 5 (Terrett et al., 1996), which has been shown to be effective in the clinical management of erectile dysfunction (Langtry and Markham, 1999) in non-diabetic and diabetic human beings (Rendell et al., 1999). Previous studies from our laboratory (Mixcotal-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001; Ambriz-Tututi et al., 2005) and from others (Jain et al., 2001, 2003; Patil et al., 2004) have consistently found that sildenafil produces antinociception in several pain models in rats and mice after local peripheral and systemic administration. However, the antinociceptive effect of this drug in diabetic rats is not well studied.

Several studies suggest that nitric oxide (NO) and cyclic GMP can activate several targets including different types of $K^+$ channels (Archer et al., 1994; Bolotina et al., 1994; Carrier et al., 1997). In line with these observations, Duarte and coworkers have recently reported that NO donors- and dibutyril

* Corresponding author. Tel.: +52 55 5061 2868; fax: +52 55 5061 2863.
*E-mail address:* vgranados@prodigy.net.mx (V. Granados-Soto).

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cyclic GMP-induced peripheral antinociception is reversed by ATP-sensitive $K^+$ channel blockers (Soares et al., 2000; Soares and Duarte, 2001), thus establishing a link between the NO–cyclic GMP pathway, opening of $K^+$ channels and antinociception. Moreover, other studies also suggest a direct relationship between central $K^+$ channels and antinociception as intrathecal administration of $K^+$ channel openers produce antinociception or increases that produced by fentanyl or clonidide (Yamazumi et al., 2001; Zushida et al., 2002). Since sildenafil accumulates cyclic GMP via inhibition of phosphodiesterase 5, this work was undertaken to determine the possible participation of the NO–cyclic GMP–protein kinase G (PKG)–$K^+$ channel pathway on spinal antinociception induced by sildenafil in non-diabetic and diabetic rats.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed on adult female Wistar rats (body weight range, 220–240 g) of 9–10 weeks of age. The animals were obtained from our own breeding facilities and had free access to drinking water, but food was withdrawn 12 h before experiments. Under this condition, we observed that streptozotocin produced a greater % of diabetic rats (90%). Experiments were done in normal light/dark cycle and they were started at the same time (10:00 AM) in the morning. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the study was approved by the Institutional Animal Care and Use Committee (Centro de Investigación y de Estudios Avanzados, México, DF, México).

### 2.2. Intrathecal surgery

Chronic catheterization of the intrathecal subarachnoid space was performed as described by Yaksh and Rudy (1976). The rats were anesthetized with a ketamine–xylazine mixture (45–12 mg/kg, i.p.), placed in a stereotaxic head holder and the atlanto-occipital membrane exposed. The latter membrane was pierced, and a polyethylene catheter (PE-10, 7.5 cm length) was inserted intrathecally and advanced caudally to the level of the thoracolumbar junction. The wound was then sutured and the animals were allowed to recover from surgery for at least 5 days before testing. Rats showing any signs of motor impairment were euthanized in a $CO_2$ chamber.

### 2.3. Induction of diabetes

Rats were intraperitoneally injected with streptozotocin (50 mg/kg) (Research Biochemical International, Natick, MA, USA) to produce experimental diabetes (Courteix et al., 1993). Control animals (age-matched) received saline 0.9%. Diabetes was confirmed 1 week after injection by measurement of tail vein blood glucose levels with the glucose meter Ascensia ELITE (Bayer, Mexico City). Two weeks after streptozotocin injection, glycemia was again determined and only animals with a final blood glucose level $\geq$300 mg/dl were included in the study (90%).

### 2.4. Measurement of antinociceptive activity

Antinociception in non-diabetic and diabetic (2 weeks) rats was assessed using the formalin test (Dubuisson and Dennis, 1977). The rats were placed in open Plexiglas observation chambers for 30 min to allow them to acclimate to their surroundings; then they were removed for formalin administration. Fifty microliters of diluted formalin (0.5% for diabetic rats or 1% for non-diabetic rats) were injected subcutaneously into the dorsal surface (Capone and Aloisi, 2004) of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the numbers of flinches of the injected paw during 1-min periods every 5 min, up to 60 min after injection (Wheeler-Aceto and Cowan, 1991). Flinching was readily discriminated and was characterized as rapid and brief withdrawal, or as flexing of the injected paw. Formalin-induced flinching behavior was biphasic (Dubuisson and Dennis, 1977). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min). Animals were used only once and at the end of the experiment they were sacrificed in a $CO_2$ chamber.

### 2.5. Examination of catheter position

At the end of the experiment, the correct position of the catheter was assessed by the intrathecal administration of 2% lidocaine (10 μl) followed by saline (10 μl) as previously described (Lozano-Cuenca et al., 2005). Motor paralysis of the hind limb of the rat occurring within 15 min after anesthetic administration was considered as an indication of correct position of the catheter. In addition, 1% methylene blue (10 μl) was injected intrathecally. The spinal cord was harvested and cut through the L4–L5 intervertebral disk to look for the catheter tip under a dissecting microscope (4× magnification). Rats showing the catheter tip positioned at sites other than the dorsal spinal cord or dye staining of paravertebral musculature were not considered for data analysis.

### 2.6. Drugs

Sildenafil citrate (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1-*H*-pyrazolo[3,4-*d*]pyrimidin-5-yl)phenyl sulfonyl]-4-methyl-piperazine) was a gift of Laboratorios Proquigama S.A. (Mexico City). *N*-L-Nitro-arginine methyl ester (L-NAME), *N*-D-nitro-arginine methyl ester (D-NAME) and 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ) were purchased from Research Biochemical International (Natick, MA, USA). KT5823 was obtained from Calbiochem (San Diego, CA, USA). Charybdotoxin, apamin and glibenclamide

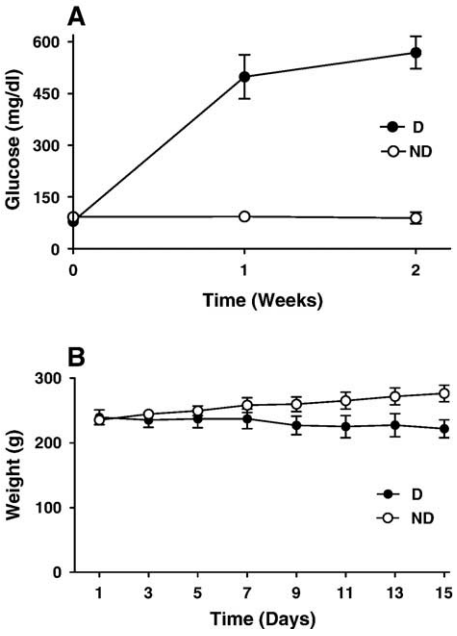


Fig. 1. Glucose levels (A) and weight (B) of rats treated with intraperitoneal streptozotocin (50 mg/kg). Data are the means±S.E.M. of eight animals. D=diabetic rats, ND=non-diabetic rats.

were purchased from Sigma (St. Louis, MO, USA). L-NAME, D-NAME, charybdotoxin, apamin and KT5823 were dissolved in saline. Sildenafil was dissolved in 20% dimethylsulfoxide (DMSO). Glibenclamide and ODQ were dissolved in 50% DMSO.

### 2.7. Study design

Non-diabetic or diabetic rats received an intrathecal injection of vehicle (20% DMSO, 10 μl) or increasing doses (12.5, 25 and 50 μg in 10 μl) of sildenafil 10 min before formalin injection into the right paw, and nociceptive behavior was assessed. To determine whether sildenafil-induced intrathecal antinociception was mediated by either the NO–cyclic GMP–PKG–$K^+$ channel pathway, effect of pretreatment (−20 min) with the appropriate vehicle (50% DMSO for glibenclamide and ODQ or saline for L-NAME, D-NAME, KT5823, charybdotoxin and apamin) or L-NAME (1–50 μg/rat), D-NAME (50 μg/rat), ODQ (1–10 μg/rat), KT5823 (5–500 ng/rat), charybdotoxin (0.01–1 ng/rat), apamin (0.1–3 ng/rat) and glibenclamide (12.5–50 μg/rat) on the antinociceptive effect induced by intrathecal sildenafil (50 μg, −10 min) was assessed. Each rat received two intrathecal injections, and appropriate controls for multiple injections and vehicles were performed before starting the formal study. Doses and drug administration schedule of drugs were selected based on previous reports (Ambriz-Tututi et al., 2005; Lozano-Cuenca et al., 2005) and on pilot experiments in our laboratory. The 10-min pretreatment for sildenafil was observed to have the best antinociceptive effect in our conditions. Observer was unaware of the treatment in each animal. Rats in all groups were observed regarding behavioral or motor function changes induced by the treatments. This was assessed, but not quantified, by testing the animals' ability to stand and walk in a normal posture, as proposed elsewhere (Chen and Pan, 2001).

### 2.8. Data analysis and statistics

All results are presented as means±S.E.M. for six to eight animals per group. Curves were made for number of flinches against time. The area under the number of flinches against time curves (AUC) for both phases of the test was calculated according to trapezoidal rule. Analysis of variance followed by Tukey's test was used to test the significance of differences between treatments. A $P<0.05$ was considered significant.

## 3. Results

### 3.1. Effect of streptozotocin

Intraperitoneal administration of streptozotocin significantly increased blood glucose levels at 1 and 2 weeks (Fig. 1A). Only these rats were included in the study. The weight of the 2-week diabetic rats became lower than those of controls by the day 9 and did not change up to day 15 (Fig. 1B). In addition, 2-week diabetic rats showed other diabetes-related symptoms like polydipsia, polyphagia and polyuria (data not shown).

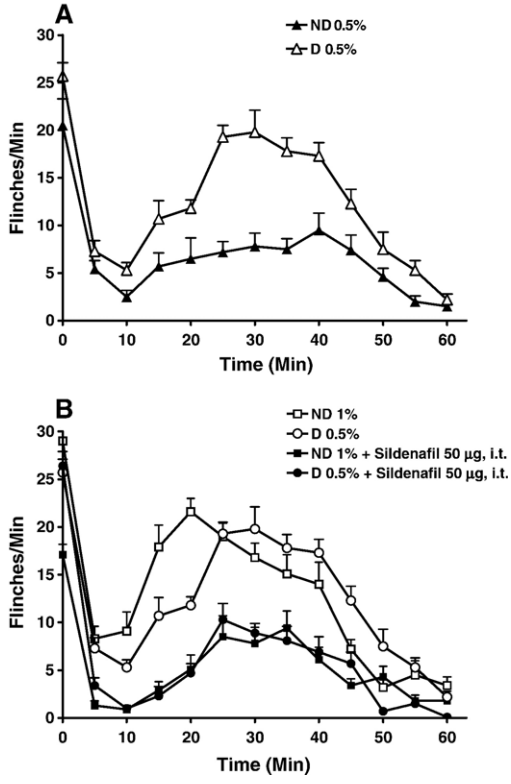


Fig. 2. (A) Time course of nociception induced by administration of 0.5% formalin to non-diabetic (ND) and diabetic (D) rats. (B) Time course of the spinal antinociceptive effect of sildenafil in rats submitted to the formalin test. Rats received an intrathecal injection of saline (−20 min) and saline or sildenafil (−10 min) pretreatment and then an injection of either 0.5% (diabetic rats, D) or 1% (non-diabetic rats, ND) formalin at time 0. Data are expressed as the mean number of flinches per min±S.E.M. of six animals.

### 3.2. Spinal antinociceptive effect of sildenafil in non-diabetic and diabetic rats

Subcutaneous formalin injection into the right hind paw of non-diabetic and diabetic (2 weeks) rats produced a typical pattern of flinching behavior characterized by a biphasic time course. Phase 1 of the nociceptive response began immediately after formalin administration and then declined gradually in approximately 10 min. Phase 2 began about 15 min after formalin administration and lasted about 1 h (Dubuisson and Dennis, 1977). Injection of 0.5% formalin into the right paw displayed a greater flinching behavior in diabetic (Fig. 2A, white triangles) compared to non-diabetic rats (Fig. 2A, black triangles). Actually, flinching behavior in 0.5% formalin-treated diabetic rats (Fig. 2B, white circles) was similar to that observed in non-diabetic rats injected with 1% formalin (Fig. 2B, white squares). Thus, both experiments suggest that the 2-week diabetes leads to hyperalgesia. The formalin-induced nociceptive behavior in non-diabetic and diabetic rats, expressed as "flinches/min", was significantly reduced by the intrathecal injection of sildenafil 50 μg given 10 min prior to formalin injection (Fig. 2B, black circles and squares). In addition, sildenafil reduced in a dose-dependent manner formalin-induced nociceptive behavior ($P<0.05$) during both phases of the test in non-diabetic (Fig. 3A and B) and diabetic (Fig. 3C and D) rats. No reduction in the reflexes was observed in either group, control or treated (data not shown).

### 3.3. Effect of the inhibition of nitric oxide synthase, guanylyl cyclase and protein kinase G on sildenafil-induced spinal antinociception in non-diabetic and diabetic rats

Intrathecal injection of L-NAME, D-NAME, ODQ and KT5823 did not produce any effect by itself. In contrast, intrathecal administration of the NO synthase inhibitor L-NAME, but not its inactive isomer D-NAME, dose-dependently reduced sildenafil-induced spinal antinociceptive activity during phases 1 (Fig. 4A) and 2 (Fig. 4B) of the test in non-diabetic rats. Contrariwise, spinal administration of L-NAME or D-NAME did not affect sildenafil-induced antinociception in diabetic rats (Fig. 4C and D). In addition, intrathecal administration of the guanylyl cyclase inhibitor ODQ (Fig. 5) or the PKG inhibitor KT5823 (Fig. 6) significantly ($P<0.05$) reduced sildenafil-induced spinal antinociceptive activity during phases 1 and 2 of the test in non-diabetic (panels A and B) and diabetic (panels C and D) rats.

### 3.4. Effect of $K^+$ channels blockers on sildenafil-induced spinal antinociception in non-diabetic and diabetic rats

The intrathecal administration of large-conductance $Ca^{2+}$-activated $K^+$ channel blocker charybdotoxin, but not saline, significantly reduced the antinociceptive activity of intrathecal sildenafil in non-diabetic rats during phases 1 (Fig. 7A) and 2 (Fig. 7B). In contrast, charybdotoxin was not able to reduce phase 1 (Fig. 7C) or phase 2 (Fig. 7D) of the formalin test in

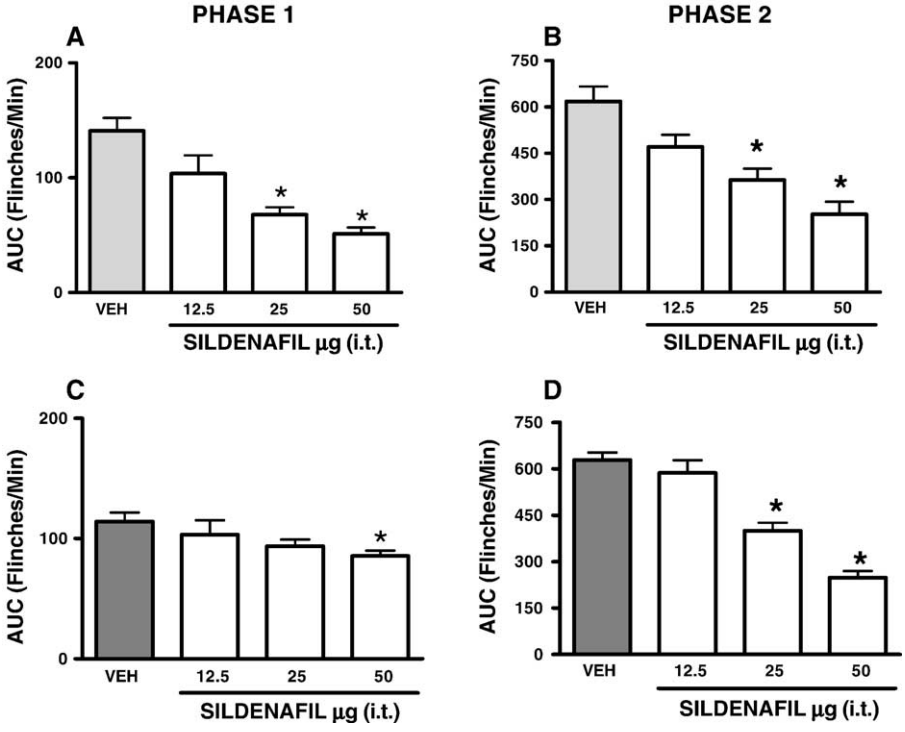


Fig. 3. Spinal antinociceptive effect of sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received an intrathecal injection of saline (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 μl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means±S.E.M. of six animals. (*) Significantly different from saline ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

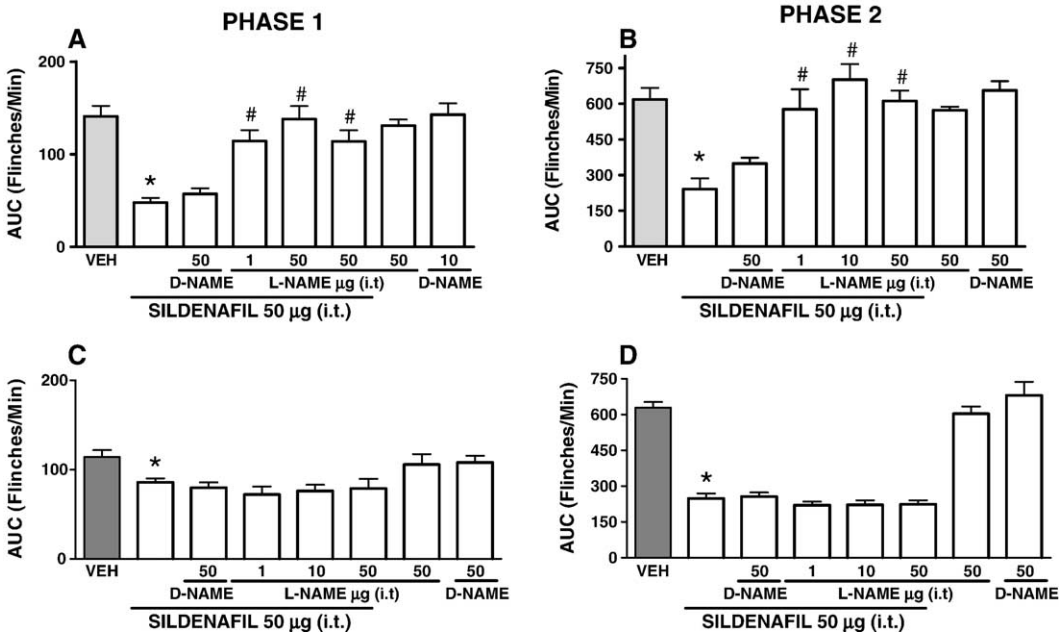


Fig. 4. Effect of *N*-L-nitro-arginine methyl ester (L-NAME) on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received an intrathecal injection L-NAME or D-NAME (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means±S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

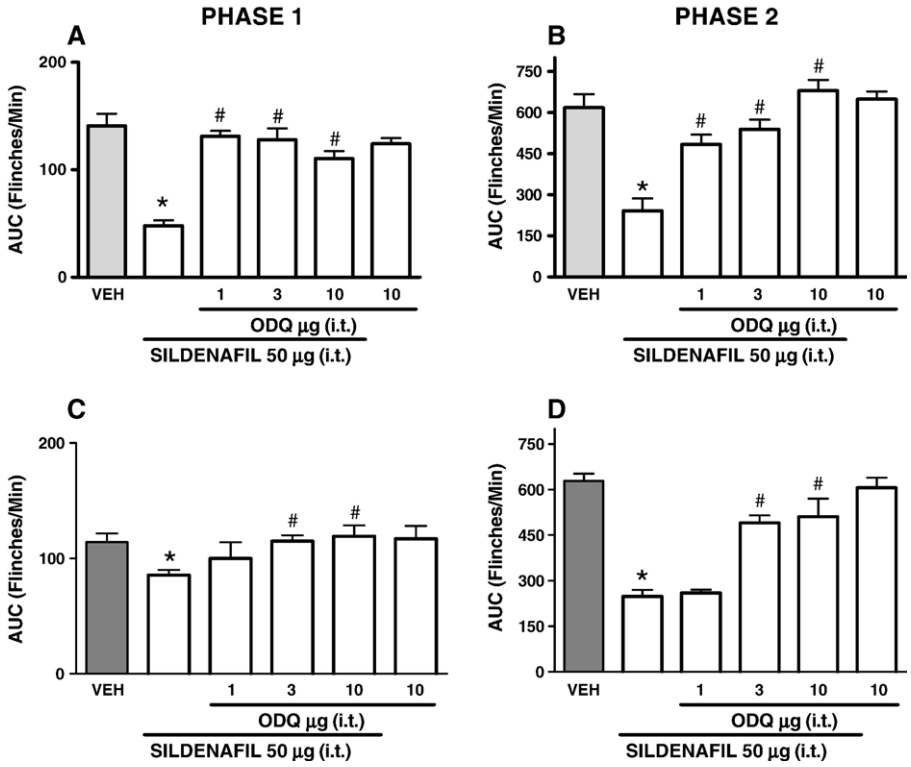


Fig. 5. Effect of 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ) on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received an intrathecal injection of ODQ (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means±S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

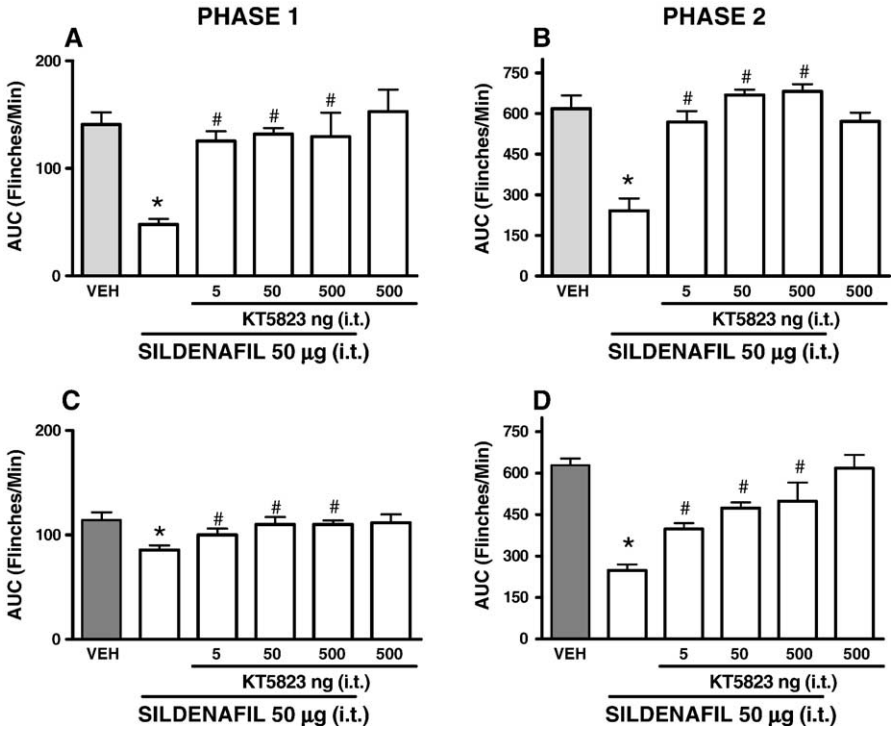


Fig. 6. Effect of KT5823 on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received a spinal injection of KT5823 (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

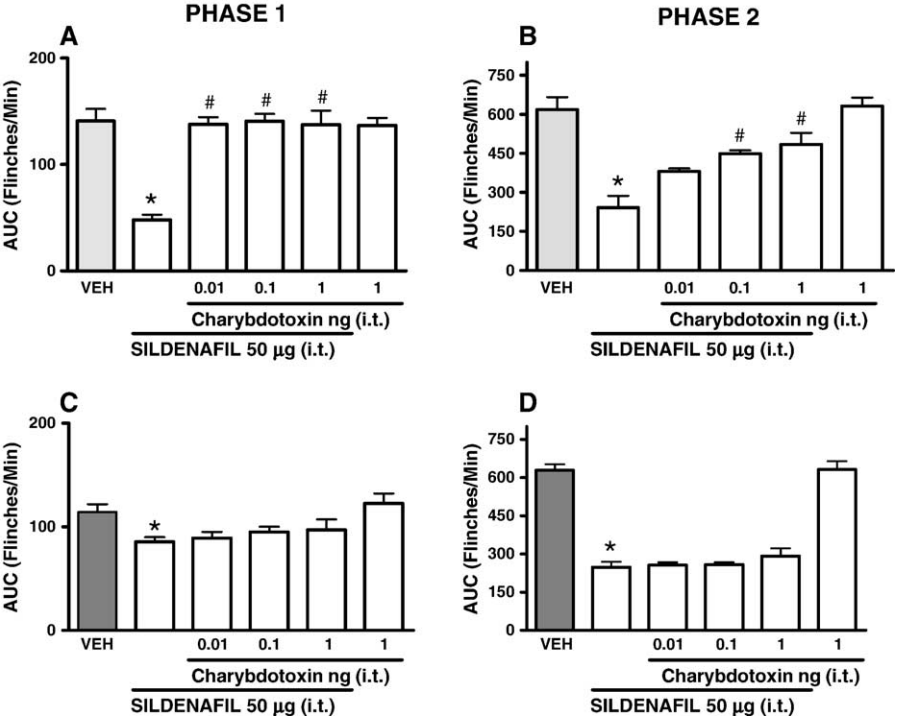


Fig. 7. Effect of charybdotoxin on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received a spinal injection of charybdotoxin (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means ± S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

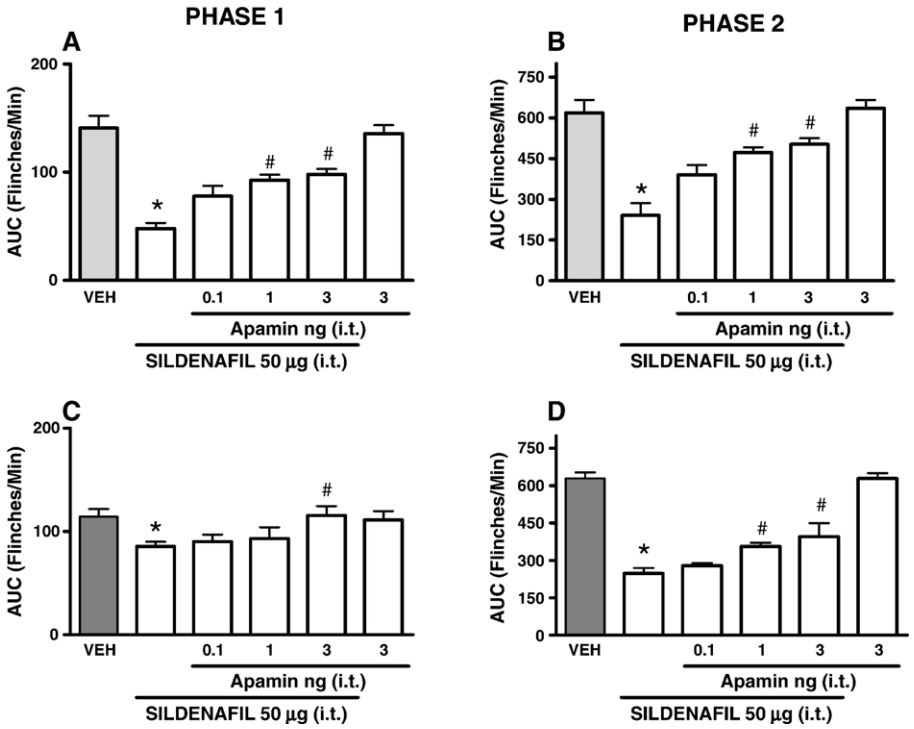


Fig. 8. Effect of apamin on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received a spinal injection of apamin (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means±S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

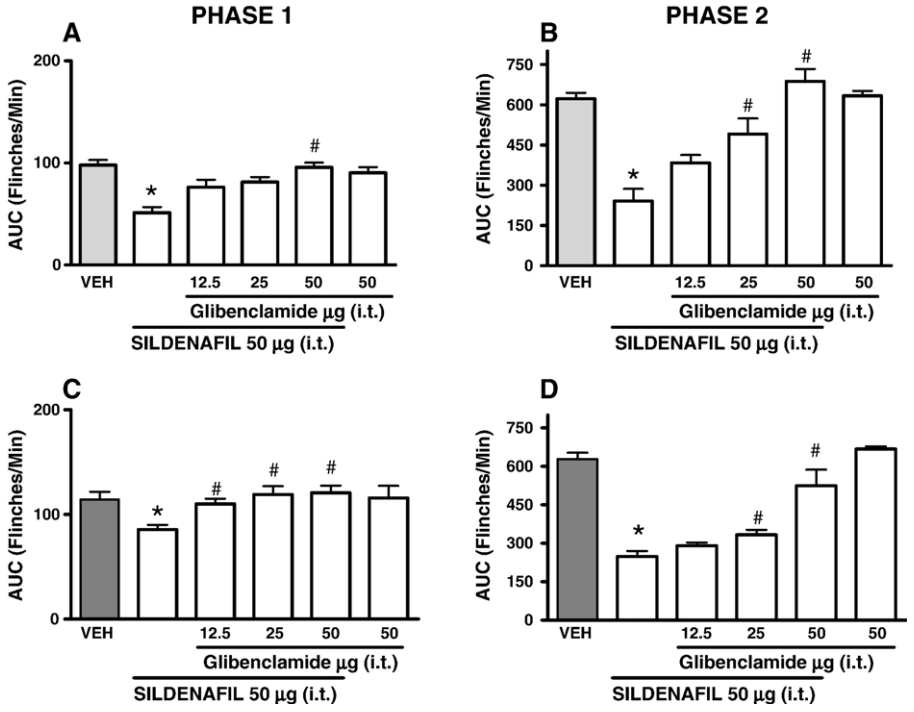


Fig. 9. Effect of glibenclamide on the spinal antinociception produced by sildenafil in non-diabetic (A and B) and diabetic (C and D) rats submitted to the formalin test. Rats received a spinal injection of glibenclamide (−20 min) and sildenafil (−10 min) pretreatment and then an injection of 0.5% (to diabetic) or 1% (to non-diabetic) formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the means±S.E.M. of six animals. (*) Significantly different from the vehicle (VEH) group ($P<0.05$) and (#) significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

diabetic rats. Apamin (small-conductance $Ca^{2+}$-activated $K^+$ channel blocker, Fig. 8) or glibenclamide (ATP-sensitive $K^+$ channel blocker, Fig. 9), but not saline, significantly reduced in a dose-dependent fashion ($P<0.05$) the antinociceptive effect of spinal sildenafil in non-diabetic (panels A and B) and diabetic (panels C and D) rats. By themselves, $K^+$ channel blockers did not affect formalin-induced nociceptive behavior in non-diabetic or diabetic rats.

## 4. Discussion

### *4.1. Diabetes-induced hyperalgesia*

Systemic administration of streptozotocin significantly increased glucose blood levels at 1 and 2 weeks and decreased weight in the rats. In addition, other diabetes-related signs (polydipsia, polyphagia and polyuria) were observed in the treated rats. These results agree with previous observations in this model (Courteix et al., 1993; Fox et al., 1999). On the other hand, 0.5% formalin injection produced a typical pattern of flinching behavior in both diabetic and non-diabetic rats. However, formalin-induced nociceptive effect was significantly higher in diabetic rats. Further, nociceptive effect induced by 0.5% formalin in diabetic rats was similar to that produced by 1% formalin injection in non-diabetic rats. Taken together, these data suggest the presence of hyperalgesia and abnormal pain processing mechanisms in the formalin test after diabetes induction (Calcutt et al., 1995, 1996; Cesena and Calcutt, 1999).

### *4.2. Antinociceptive action of sildenafil in non-diabetic and diabetic rats*

We have previously reported that sildenafil (an inhibitor of phosphodiesterase 5) by itself is able to produce peripheral antinociception in the formalin test (Mixcotal-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001; Ambriz-Tututi et al., 2005). These findings have been confirmed in the acetic acid-induced nociception and carrageenan-induced hyperalgesia after peripheral and systemic administration (Jain et al., 2001, 2003). In addition, we have reported that intrathecal administration of sildenafil is also effective in the formalin test (Torres-López et al., 2002). In the present study, we confirmed our former observation that sildenafil is able to produce spinal antinociception in non-diabetic rats using the formalin test. However, this study extends those observations by showing a spinal antinociceptive effect for this phosphodiesterase 5 inhibitor in 2-week diabetic rats. Our study agrees with previous data on the local peripheral and systemic antinociceptive effect of sildenafil in 8-week diabetic rats in the mouse writhing test and Randall-Sellito test in the rat (Patil et al., 2004). We observed that sildenafil significantly reduced in a similar way flinching behavior during phase 2 in non-diabetic and diabetic rats. In contrast, the antinociceptive effect of sildenafil during phase 1 was more evident in non-diabetic compared to diabetic rats. This difference could be due to the different level of nociception reached in both conditions (i.e. non-diabetic vs. diabetic) as number of flinches was greater in non-diabetic rats.

Sildenafil is a potent, selective and reversible phosphodiesterase 5 inhibitor that blocks cyclic GMP hydrolysis (Terrett et al., 1996; Moreland et al., 1999). Therefore, our results suggest a significant participation of spinal phosphodiesterase 5 in non-diabetic and diabetic rats. This suggestion is in line with evidence showing that phosphodiesterase 5 is expressed in almost all rat lumbar spinal neurons (Nakamizo et al., 2003). Thus, these data indicate that inhibition of phosphodiesterase 5 and therefore the accumulation of cyclic GMP produces intrathecal antinociception in the formalin test. Cyclic GMP has several targets to produce its effects in cells (Lucas et al., 2000), including cyclic GMP-dependent protein kinases (PKG), cyclic GMP-regulated phosphodiesterases and cyclic nucleotide-gated ion channels. In addition, cyclic GMP is a component of the NO–cyclic GMP–PKG–$K^+$ channels pathway, which plays an important role in peripheral antinociception (Sachs et al., 2004; Ambriz-Tututi et al., 2005). We have assessed the possible participation of this pathway on sildenafil-induced intrathecal antinociception in non-diabetic and diabetic rats in order to gain further insight in the spinal mechanism of action of sildenafil.

### *4.3. Effect of the inhibition of nitric oxide synthase, guanylyl cyclase and protein kinase G on sildenafil-induced spinal antinociception in non-diabetic and diabetic rats*

Intrathecal administration of the NO synthase inhibitor L-NAME dose-dependently reduced sildenafil-induced intrathecal antinociceptive activity in non-diabetic, but not in diabetic, rats. In addition, D-NAME, the inactive isomer of L-NAME, was not able to modify sildenafil-induced intrathecal antinociception in non-diabetic or diabetic rats. The intrathecal administration of the guanylyl cyclase and PKG inhibitors significantly reduced the intrathecal antinociceptive effect of sildenafil in both non-diabetic and diabetic rats. So far, there are no reports about the effect of the intrathecal administration of nitric oxide synthesis, guanylyl cyclase and PKG inhibitors on the intrathecal antinociceptive activity of sildenafil. However, these results agree with previous reports showing that sildenafil-induced systemic antinociception can be blocked by the inhibitor of the guanylyl cyclase methylene blue in both non-diabetic and diabetic rats (Patil et al., 2004). However, they disagree with those reporting that L-NAME was able to reverse the systemic antinociceptive effect of sildenafil in the writhing test in diabetic mice (Patil et al., 2004). Notwithstanding, our results agree with previous observations showing that L-NAME and ODQ are able to reduce lumiracoxib- or L-arginine-induced antinociception (Lozano-Cuenca et al., 2005; Kamei et al., 2005). Our data suggest that a NO–cyclic GMP–PKG spinal pathway is involved in sildenafil-induced intrathecal antinociception in non-diabetic rats. Accordingly, it has been reported the presence of all components of this pathway in the spinal cord (Tao and Johns, 2002; Tegeder et al., 2002). Although matter of debate, it has been reported that NO can decrease the mechanical responsiveness of nociceptors and its action might depend on the baseline level of neuronal excitability (Levy and Strassman, 2004). Moreover, an analogue of cyclic GMP produced inhibition of spontaneous activity and mechanical

responses of nociceptive afferents (Levy and Strassman, 2004; Liu et al., 2004). Contrariwise, the intrathecal antinociceptive effect of diclofenac or ibuprofen in glutamate-induced hyperalgesia was reversed by intrathecal L-arginine, but not by D-arginine (Björkman, 1995), thus suggesting that these NSAIDs could act via the functional inhibition of the pronociceptive actions of NO. In line with this observation, but contrary to our results, intrathecal administration of L-NAME produced antinociception in the formalin test (Malmberg and Yaksh, 1993). Differences observed between these reports and our results could be due to the type and intensity of the noxious stimuli, rat strain and particularly to the dose or concentration reached at the active site (Granados-Soto, 2003). In this sense, evidence suggests that low doses are associated with antinociception, whereas that medium or high doses of NO produce nociception or no effect (Sousa and Prado, 2001; Kina et al., 2005).

Our results suggest that diabetes may induce changes leading to a reduction in activity or expression of NO synthase at the spinal cord. Accordingly, expression of neuronal NO synthase is reduced in dorsal root ganglion of 8-week diabetic rats (Sasaki et al., 1998; Yasuda, 1999). Therefore, the observed lack of effect of L-NAME to reverse sildenafil-induced spinal antinociception in diabetic, but not in non-diabetic, rats could be due to a reduced activity or expression of NO synthase at the spinal cord. In line with this suggestion, we have observed a reduction in the activity of NO synthase in 2-week diabetic rats (data not shown). The fact that ODQ and KT5823 were still able to reduce the spinal antinociceptive effect of sildenafil in diabetic rats suggests that cyclic GMP–PKG pathway was not modified by diabetes. However, there is evidence reporting a reduction of cyclic GMP content in dorsal root ganglion of 8-week diabetic rats (Sasaki et al., 1998). Differences could be due to different times of diabetes induction, as this work was done in 2-week diabetic rats. Taken together, data suggest that activation of the spinal NO–cyclic GMP–PKG pathway is involved in sildenafil-induced spinal antinociception in non-diabetic rats. The first step of this pathway (NO), but not second and third, seems to be reduced in diabetic rats of 2 weeks.

### *4.4. Effect of $K^+$ channels blockers on sildenafil-induced spinal antinociception in non-diabetic and diabetic rats*

The results obtained suggest that modulation of $K^+$ channels at the spinal level may represent an important step in the mechanism of spinal antinociception induced by sildenafil in non-diabetic and diabetic rats. Intrathecal administration of glibenclamide (ATP-sensitive $K^+$ channel blocker, Davies et al., 1991; Edwards and Weston, 1993), apamin and charybdotoxin (small- and large-conductance $Ca^{2+}$-activated $K^+$ channel blockers, respectively; Romey et al., 1984; Stretton et al., 1992) significantly reduced the antinociceptive action of sildenafil in non-diabetic rats, suggesting that this phosphodiesterase 5 inhibitor activates ATP-sensitive, small- and large-conductance $Ca^{2+}$-activated $K^+$ channels at spinal sites. This observation agrees with a previous report about the participation of $K^+$ channels on sildenafil-induced peripheral antinociception (Ambriz-Tututi et al., 2005). These data are also according with the presence of several types of $K^+$ channels in spinal cord dorsal horn neurons (Yamashita et al., 1994; Safronov, 1999; Mongan et al., 2005) and with the intrathecal antinociceptive effect of $K^+$ channel openers (Yamazumi et al., 2001; Zushida et al., 2002).

Our group (Lázaro-Ibáñez et al., 2001; Ortiz et al., 2003; Ambriz-Tututi et al., 2005) and others (Soares et al., 2000; Soares and Duarte, 2001; Sachs et al., 2004) have reported that drugs which activate the NO–cyclic GMP pathway seem to modulate the opening of $K^+$ channels in order to produce antinociception. In the current study, it has been observed that sildenafil-induced spinal antinociceptive effect was significantly reduced by L-NAME, ODQ and KT5823, thus suggesting that, at spinal sites, sildenafil is able to activate the NO–cyclic GMP–PKG pathway. In addition, the spinal antinociception of sildenafil was blocked by glibenclamide, apamin and charybdotoxin suggesting that sildenafil also activates $K^+$ channels (see above). Taken together, these data suggest that the reduction of nociception produced by sildenafil in non-diabetic rats could be due to the activation of the NO–cyclic GMP–PKG–$K^+$ channel spinal pathway. The fact that sildenafil is able to relax the ductus arteriosus (Thébaud et al., 2002) and clitoris (Gragasin et al., 2004) by increasing cyclic GMP levels and thereby activating the cyclic GMP–PKG–$K^+$ channel pathway to produce membrane hyperpolarization is in line with our data.

Our results suggest that diabetes may induce changes related with a reduction in the function of large-conductance $Ca^{2+}$-activated $K^+$ channels as charybdotoxin was not able to reverse sildenafil-induced spinal antinociception in the formalin test in diabetic rats. In line with these results, it has been reported that activation of $Ca^{2+}$-activated $K^+$ channels produces less cerebral vasodilatation in diabetic compared to non-diabetic rats (Mayhan et al., 2004). Taken together, these data suggest that diabetes may have a deleterious effect on the function of some types of $K^+$ channels. There is evidence showing that the function and expression of ATP- and voltage-sensitive $K^+$ channels is also reduced in diabetic rats (Kamei et al., 1994; Sood et al., 2000). However, in our conditions, sildenafil-induced intrathecal antinociception was not affected by the ATP-sensitive $K^+$ channel inhibitor glibenclamide or the small-conductance $Ca^{2+}$-activated $K^+$ channel inhibitor apamin in diabetic rats. These differences could be explained by the different protocols to induce diabetes as well as the different species used in the studies.

In summary, intrathecal administration of sildenafil produced antinociception in non-diabetic and diabetic rats. The mechanisms underlying sildenafil-induced intrathecal antinociception in non-diabetic rats could be due to the increase in cyclic GMP concentration, which in turn would activate PKG; this event would lead to opening of several types of $K^+$ channels, hyperpolarization of the central terminal of primary afferent neurons and finally antinociception. Notwithstanding the changes in the function of NO synthase and high-conductance $Ca^{2+}$-activated $K^+$ channels induced by diabetes, sildenafil was

still able to induce spinal antinociception in diabetic rats suggesting that this drug could have a role in the pharmacotherapy of pain associated with this process.

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