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European Journal of Pharmacology 512 (2005) 121–127

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Analysis of the mechanism underlying the peripheral antinociceptive action of sildenafil in the formalin test

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Received 16 September 2004; received in revised form 24 January 2005; accepted 31 January 2005
Available online 31 March 2005

Abstract

The mechanism of the antinociceptive action of the phosphodiesterase 5 inhibitor, sildenafil, was assessed in the formalin test. Local peripheral ipsilateral, but not contralateral, administration of sildenafil (50–200 μg/paw) produced a dose-related antinociception during both phases of the formalin test. The local peripheral pretreatment with protein kinase G inhibitor peptide (PKG inhibitor, 0.01–1 μg/paw), charybdotoxin (large- and intermediate-conductance Ca^{2+}-activated K^+ channel blocker, 0.01–1 μg/paw), apamin (small-conductance Ca^{2+}-activated K^+ channel blocker, 0.1–2 μg/paw), tolbutamide (ATP-sensitive K^+ channel blocker, 12.5–50 μg/paw), and tetraethylammonium (non-selective voltage-dependent K^+ channel blocker, 12.5–50 μg/paw), but not 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ, inhibitor of guanylyl cyclase, 12.5–50 μg/paw) or saline, significantly diminished in a dose-dependent manner sildenafil-induced local peripheral antinociception. Given alone, local peripheral administration of inhibitors did not modify formalin-induced nociceptive behavior. Results suggest that sildenafil produces its local peripheral antinociceptive effect via activation of the cyclic GMP–PKG–K^+ channel pathway.

Keywords: Sildenafil; Cyclic GMP; Protein kinase G; K^+ channel; Peripheral processing

1. Introduction

Sildenafil (Viagra®) 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). Previous studies from our laboratory (Mixcoatl-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001) and from others (Jain et al., 2001, 2003) have consistently found that sildenafil produces antinociception in several pain models in rats and mice after local peripheral and systemic administration. We have suggested that sildenafil could produce its antinociceptive effect through the accumulation of cyclic GMP as a consequence of phosphodiesterase 5 inhibition (Mixcoatl-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001). Accordingly, there is evidence that local peripheral (Soares and Duarte, 2001; Alves et al., 2004) or spinal (Tegeder et al., 2002) cyclic GMP analogues are able to produce antinociception in several models of inflammatory pain.

Several studies suggest that diclofenac-induced peripheral antinociception is produced through the activation of the nitric oxide (NO)–cyclic GMP pathway (Tonussi and Ferreira, 1994; Ortiz et al., 2003a; Alves et al., 2004). Like diclofenac, other non-steroidal anti-inflammatory drugs (NSAIDs) modulate the NO–cyclic GMP pathway at the periphery (Duarte et al., 1990, 1992; Ferreira et al., 1991;

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doi:10.1016/j.ejphar.2005.01.055

Granados-Soto et al., 1995; Islas-Cadena et al., 1999; Aguirre-Bañuelos and Granados-Soto, 2000; Lázaro-Ibáñez et al., 2001; Ortiz et al., 2003a,b; Alves et al., 2004). 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 et al. have recently reported that NO donors- and dibutyryl 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 intracerebroventricular administration of K^+ channel openers produces antinociception in rats and mice (Narita et al., 1993), whereas intracerebroventricular or intrathecal K^+ channel blockers reduce morphine- or fentanyl-induced antinociception (Ocana et al., 1990; Yamazumi et al., 2001). Based on the above considerations, this work was undertaken to determine the possible participation of the cyclic GMP–PKG–K^+ channel pathway on peripheral antinociception induced by sildenafil. For this purpose, we tested the actions of 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ, an inhibitor of guanylyl cyclase) (Moro et al., 1996), PKG inhibitor peptide (an inhibitor of PKG; Glass, 1983), charybdotoxin (an inhibitor of intermediate- and large-conductance Ca^{2+}-activated K^+ channels; Stretton et al., 1992), apamin (an inhibitor of small-conductance Ca^{2+}-activated K^+ channels; Romey et al., 1984), tolbutamide (ATP-sensitive K^+ channel blockers; Edwards and Weston, 1993), and tetraethylammonium (voltage-dependent K^+ channel inhibitors; Cook and Quast, 1990) on sildenafil-induced local peripheral antinociception in the 1% formalin test.

2. Materials and methods

2.1. Animals

Experiments were performed on adult female Wistar rats (body weight range, 180–220 g) of 6–7 weeks of age. Female rats were used based on the fact that previous experiments in our conditions (Wistar rats, formalin concentration of 1%, and weight range of 180–220 g) have not shown significant differences between males and females (unpublished data). Other authors have found differences only with other rat strains, greater weight, or different formalin concentrations (Gaumond et al., 2002). The animals were obtained from our own breeding facilities and had free access to food and drinking water before experiments. 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. Measurement of antinociceptive activity

Antinociception 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 (1%) was injected s.c. 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 number 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 (cf. 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). At the end of the experiment, the rats were sacrificed in a CO_2 chamber.

2.3. 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 SA (Mexico City). 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ) was purchased from Research Biochemical International (Natick, MA, USA). Charybdotoxin, apamin, tolbutamide, and tetraethylammonium were purchased from Sigma (St. Louis, MO, USA). The protein kinase G (PKG) inhibitor peptide (Arg-Lys-Arg-Ala-Arg-Lys-Glu) was obtained from Calbiochem (San Diego, CA, USA). Sildenafil, charybdotoxin, apamin, PKG inhibitor, and tetraethylammonium were dissolved in saline. Tolbutamide and ODQ were dissolved in 20% dimethylsulfoxide (DMSO).

2.4. Study design

Rats received a s.c. injection (50 μl) into the dorsal surface of the right hind paw of vehicle (20% DMSO or saline) or increasing doses (50, 100, and 200 μg) of sildenafil 20 min before formalin injection into the ipsilateral paw. To determine whether sildenafil acted locally, this drug was administered to the left (contralateral) paw 20 min before formalin was injected into the right paw, and the corresponding effect on nociceptive behavior was assessed. To determine whether sildenafil-induced peripheral anti-

nociception was mediated by the cyclic GMP–PKG–K^+ channel pathway, the effect of pretreatment (− 10 min) with the appropriate vehicle (20% DMSO for ODQ and tolbutamide or saline for charybdotoxin, apamin, PKG inhibitor, and tetraethylammonium), ODQ (12.5–50 μg/paw), PKG inhibitor (0.01–1 μg/paw), charybdotoxin (0.01–1 μg/paw), apamin (0.1–2 μg/paw), tolbutamide (12.5–50 μg/paw), or tetraethylammonium (12.5–50 μg/paw) on the antinociceptive effect induced by local peripheral sildenafil (200 μg/paw) was assessed. Each rat received a total of three injections: one of sildenafil or vehicle, another of the guanylyl cyclase inhibitor, PKG inhibitor, or K^+ channel blockers, and other of formalin. Doses and drug administration schedule of drugs were selected based on previous reports (Rodrigues and Duarte, 2000; Mixcoatl-Zecuatl et al., 2000; Soares et al., 2000; Soares and Duarte, 2001; Asomoza-Espinosa et al., 2001) and on pilot experiments in our laboratory. The 20 min pretreatment for sildenafil was observed to have the best antinociceptive effect in our conditions. The observer was unaware of the treatment given to each animal. Rats in all groups were tested for possible side effects such as reduction of righting, stepping, and corneal and pinna reflexes, before and after drug treatment.

2.5. Data analysis and statistics

All results are presented as mean ± S.E.M. for six 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 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. Peripheral and spinal antinociceptive effects of sildenafil

Subcutaneous formalin injection into the right hind paw produced a typical pattern of flinching behavior characterized by a biphasic time course (Fig. 1A, open circles). 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 (cf. Dubuisson and Dennis, 1977; Porro and Cavazzuti, 1993). The formalin-induced nociceptive behavior, expressed as "mean flinches per minute," was significantly reduced by the ipsilateral local peripheral injection of sildenafil, 200 μg, given 20 min prior to formalin injection (Fig. 1A, black circles). In addition, sildenafil reduced in a dose-dependent manner formalin-induced nociceptive behavior ($P<0.05$) during both phase 1 (Fig. 1A and B) and phase 2 (Fig. 1C) of the test. Contralateral injection of sildenafil, 200 μg, was ineffective (Fig. 1B and C). No reduction in the assessed reflexes was observed in either group, control or treated (data not shown).

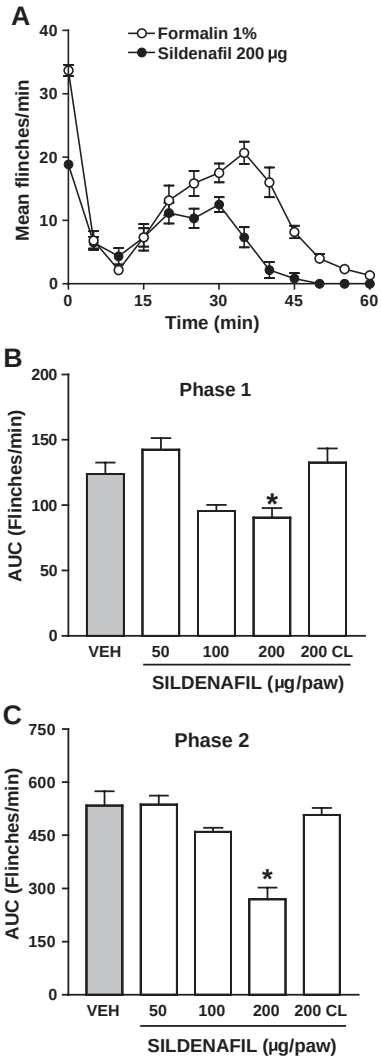

Fig. 1. Time course of the local peripheral antinociceptive effect of sildenafil in the formalin test (Panel A). Dose–response relationship obtained after local peripheral administration of sildenafil (Panel B) during phase 2 of the formalin test. In both cases, rats were pretreated with an intraplantar injection of saline or sildenafil into either the right or left (contralateral, CL) paw, 20 min before formalin injection. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. of six animals. *Significantly different from saline ($P<0.05$), as determined by analysis of variance followed by Tukey's test.

3.2. Effect of the inhibition of guanylyl cyclase and protein kinase G on sildenafil-induced antinociception

The local peripheral administration of the inhibitor of guanylyl cyclase ODQ did not produce any effect by itself nor affected sildenafil-induced peripheral antinociception

(Fig. 2A). In contrast, the local peripheral administration of the PKG inhibitor was not able to reduce formalin-induced nociceptive behavior, but this peptide significantly diminished peripheral antinociception induced by sildenafil ($P<0.05$; Fig. 2B).

3.3. Effect of K^+ channels blockers on sildenafil-induced antinociception

The local peripheral administration of K^+ channel blockers charybdotoxin (large- and intermediate-conductance Ca^{2+}-activated K^+ channel blocker; Fig. 3A), apamin (small-conductance Ca^{2+}-activated K^+ channel blocker; Fig. 3B), tolbutamide (ATP-sensitive K^+ channel blocker; Fig. 4A), and tetraethylammonium (voltage-gated K^+ channel blocker; Fig. 4B), but not saline, significantly reduced in a dose-dependent fashion ($P<0.05$) the antinociceptive activity of local peripheral sildenafil. By themselves, K^+ channel blockers did not affect formalin-induced nociceptive behavior.

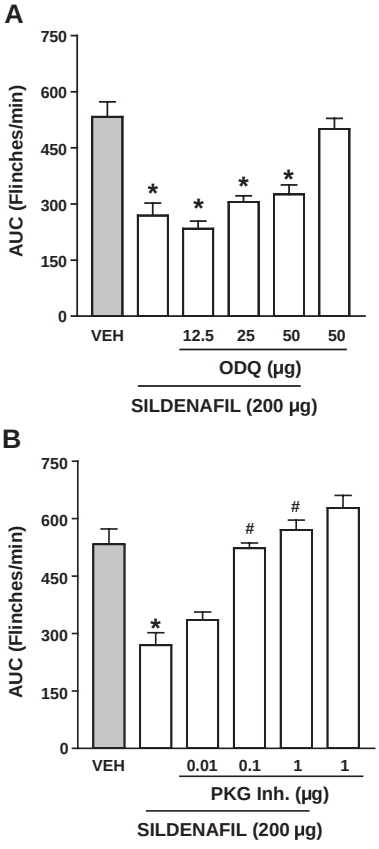

Fig. 2. Effect of 1*H*-(1,2,4)-oxadiazolo(4,2-*a*)quinoxalin-1-one (ODQ; Panel A) and PKG inhibitor peptide (PKG Inh.; Panel B) on the local peripheral antinociception produced by sildenafil on the second phase of the formalin test. Rats received a local peripheral injection of sildenafil, 200 µg (− 20 min), and either ODQ or PKG inhibitor pretreatment (− 10 min) and then an injection of 1% formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. of six animals. *Significantly different from the vehicle (VEH) group ($P<0.05$). #Significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

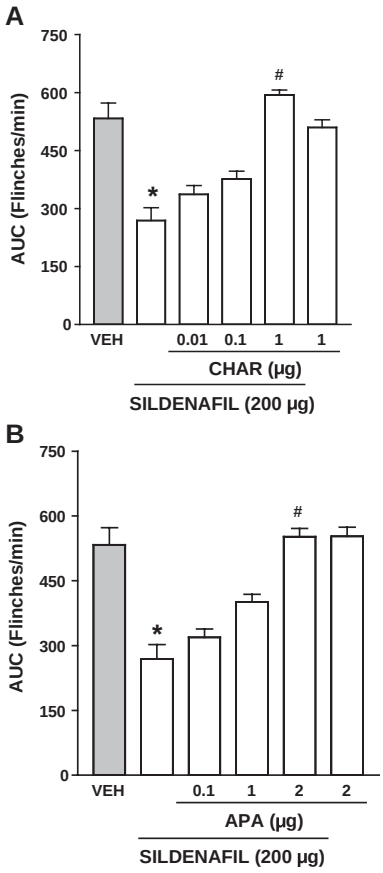

Fig. 3. Effect of charybdotoxin (CHAR; Panel A) and apamin (APA; Panel B) on the local peripheral antinociception produced by sildenafil during the second phase of the formalin test. Rats received a local peripheral injection of sildenafil, 200 µg (− 20 min), and either charybdotoxin or apamin pretreatment (− 10 min) and then an injection of 1% formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. of six animals. *Significantly different from the vehicle (VEH) group ($P<0.05$). #Significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

4. Discussion

4.1. Antinociceptive action of sildenafil

As previously reported (Mixcoatl-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001), in this work, we have found that local peripheral administration of sildenafil (an inhibitor of phosphodiesterase 5) is able to produce antinociception in both phases of the formalin test. 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; Patil et al., 2003). Based on the fact that sildenafil is a potent, selective, and reversible phosphodiesterase 5 inhibitor (Terrett et al., 1996; Moreland et al., 1999) that

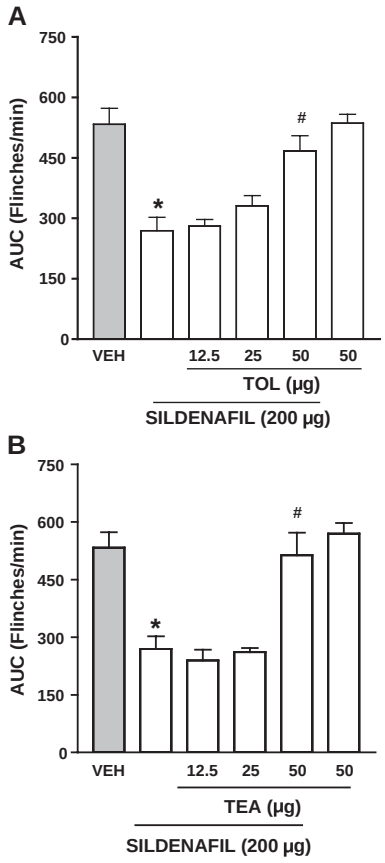

Fig. 4. Effect of tolbutamide (TOL; Panel A) and tetraethylammonium (TEA; Panel B) on the local peripheral antinociception produced by sildenafil during the second phase of the formalin test. Rats received a local peripheral injection of sildenafil, 200 µg (− 20 min), and either tolbutamide or tetraethylammonium pretreatment (− 10 min) and then an injection of 1% formalin (50 µl) at time 0. Data are expressed as the area under the number of flinches against time curve (AUC). Bars are the mean ± S.E.M. of six animals. *Significantly different from the vehicle (VEH) group ($P<0.05$). #Significantly different from the sildenafil group ($P<0.05$), as determined by analysis of variance followed by the Tukey's test.

blocks cyclic GMP hydrolysis, we previously suggested that sildenafil could produce its peripheral antinociceptive effect through the increase of intracellular cyclic GMP levels (Mixcoatl-Zecuatl et al., 2000; Asomoza-Espinosa et al., 2001). In addition, our results suggest a significant participation of peripheral phosphodiesterase 5. This suggestion is in line with evidence showing that phosphodiesterase 5 is expressed in several tissues (Beavo, 1995; Moreland et al., 1999). Thus, these data indicate that inhibition of phosphodiesterase 5 and therefore the accumulation of cyclic GMP produce peripheral 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. Of these, we have assessed the possible participation of PKG and K^+ channels on sildenafil-induced peripheral antinociception.

4.2. Effect of the inhibition of guanylyl cyclase and PKG on sildenafil-induced antinociception

The local peripheral administration of ODQ did not produce any effect on sildenafil-induced peripheral antinociception. Since ODQ blocks soluble guanylyl cyclase (Moro et al., 1996), our results suggests the lack of participation of this protein on the antinociceptive effect of sildenafil. Contrary to our results, other authors have reported that methylene blue, another guanylyl cyclase inhibitor, was able to reverse the antinociceptive effect of sildenafil after local peripheral and systemic administration (Jain et al., 2001). However, this seems unlikely as cyclic GMP increase is probably produced by inhibition of phosphodiesterase 5, but not by the ongoing activity of guanylyl cyclase.

As observed in other systems (Archer et al., 1994; Hampl et al., 1995; Tanaka et al., 1998; Thébaud et al., 2002), we hypothesized that cyclic GMP accumulation induced by inhibition of phosphodiesterase 5 would produce activation of PKG, which in turn would phosphorylate K^+ channels and then antinociception. In line with this suggestion, the PKG inhibitor reduced in a dose-dependent manner sildenafil-induced antinociception. Similar observations have been recently reported as the PKG inhibitor KT5823 reduced the antinociceptive effect of drugs which activate the NO–cyclic GMP pathway (morphine, metamizol, and *S*-nitroso-*n*-acetyl-D,L-penicilamine) (Sachs et al., 2004). Taken together, these results suggest that cyclic GMP, increased by sildenafil, may indeed activate PKG as an intermediate step to finally produced peripheral antinociception.

4.3. Effect of the blockade of potassium channels on sildenafil-induced antinociception

The peripheral antinociceptive effect of sildenafil was blocked by local peripheral pretreatment with charybdotoxin and apamin (large- and small-conductance Ca^{2+}-activated K^+ channel inhibitors, respectively; Romey et al., 1984; Stretton et al., 1992), suggesting the possible participation of both types of Ca^{2+}-activated K^+ channels on sildenafil-induced antinociceptive effect. Tolbutamide, an ATP-sensitive K^+ channel inhibitor (Edwards and Weston, 1993), also reduced the antinociceptive effect of sildenafil, thus suggesting that sildenafil may activate this channel in order to reduce pain in the formalin test. It is known that besides its effect on large-conductance Ca^{2+}-activated K^+ channel, charybdotoxin is also able to inhibit intermediate-conductance Ca^{2+}-activated and voltage-gated K^+ channels (Price et al., 1989; Ouadid-Ahidouch et al., 1999). Therefore, blockade of sildenafil-induced antinociception by apamin, charybdotoxin, and tolbutamide suggests that sildenafil may be producing its antinociceptive effect through activation of small-, intermediate-, and large-conductance Ca^{2+}-activated K^+ channel as well as

voltage-gated K^+ channels. The reduction of the antinociceptive effect of sildenafil by tetraethylammonium, a non-selective voltage-gated K^+ channel inhibitor (Cook and Quast, 1990), agrees with this suggestion.

Duarte et al. have reported that sodium nitroprusside- or dibutyryl cyclic GMP-induced antinociception is blocked by tolbutamide and glibenclamide, but not by apamin, charybdotoxin, 4-aminopyridine, or tetraethylammonium, thus suggesting the possible participation of ATP-sensitive K^+ channels on NO- and cyclic GMP-induced peripheral antinociception (Soares and Duarte, 2001; Soares et al., 2000). Our data agree with these results suggesting the participation of ATP-sensitive K^+ channels. However, our work also suggests the possible participation of small-, intermediate-, and large-conductance Ca^{2+}-activated K^+ channels as well as voltage-gated K^+ channels. Differences could be due to different models of pain used. Our results are similar to those previously reported by our group, as peripheral antinociception induced by drugs which activate the NO–cyclic GMP pathway can be blocked by several types of K^+ channel blockers, suggesting the participation of ATP-sensitive, voltage-gated, small-, intermediate-, and large-conductance Ca^{2+}-activated K^+ channels (Lázaro-Ibáñez et al., 2001; Ortiz et al., 2002, 2003a,b). The fact that sildenafil dilates the ductus arteriosus by increasing cyclic GMP levels and thereby activating Ca^{2+}-sensitive K^+ channel and membrane hyperpolarization (Thébaud et al., 2002) is in line with our data.

In summary, local peripheral administration of sildenafil produced antinociception in the formalin test. The mechanisms underlying sildenafil-induced peripheral antinociception 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 primary afferent neurons, and finally antinociception.

Acknowledgements

The authors greatly appreciate the technical and bibliographic assistance of Guadalupe C. Vidal-Cantú and Hector Vázquez, respectively. This work was partially supported by a UMSNH grant. Vinicio Granados-Soto is the recipient of a sabbatical fellowship from CONACYT.

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