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European Journal of Pharmacology 489 (2004) 59–65
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Additive antinociceptive effect of the combination of diazoxide, an activator of ATP-sensitive K^+ channels, and sodium nitroprusside and dibutyryl-cGMP

Daniela P. Alves, Adriana C. Soares, Janetti N. Francischi, Maria S.A. Castro, Andréa C. Perez, Igor D.G. Duarte*

Department of Pharmacology, Institute of Biological Sciences, UFMG, Av. Antônio Carlos, 6622-Campus da Pampulha, CEP: 31.270.100, Belo Horizonte MG, Brazil

Received 26 November 2003; received in revised form 3 February 2004; accepted 10 February 2004

Abstract

Using the rat paw pressure test, in which increased sensitivity is induced by intraplantar injection of prostaglandin E_2, we assessed the antinociceptive effect of the ATP-sensitive K^+ channel opener diazoxide and the large-conductance Ca^{2+}-activated K^+ channel opener NS-1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) on the peripheral hyperalgesia induced by prostaglandin E_2. Diazoxide, administered locally into the right hindpaw (20, 38, 75, 150, 300 and 600 μg), elicited a dose-dependent antinociceptive effect on prostaglandin E_2-induced hyperalgesia (2 μg/paw). The effect of diazoxide at the dose of 300 μg/paw was shown to be local since it did not produce any effect when administered in the contralateral paw. The action of diazoxide (300 μg/paw) as an ATP-sensitive K^+ channel opener seems to be specific, since its effect was antagonized in a dose-dependent manner by glibenclamide (40, 80 and 160 μg/paw), a specific blocker of these channels, while tetraethylammonium (7.5, 15 and 30 μg/paw), dequalinium (12.5, 25 and 50 μg/paw) or charybdotoxin (0.5, 1 and 2 μg/paw), blockers of voltage-dependent K^+ channels and of small- and large-conductance Ca^{2+}-activated K^+ channels, respectively, were not able to abolish the antinociception induced by diazoxide. The peripheral antinociceptive effect of diazoxide was not prevented by prior administration of naloxone (12.5, 25 and 50 μg/paw), an opioid receptor antagonist, or methylene blue (75, 125 and 300 μg/paw), an agent that inhibits the activation of guanylate cyclase by nitric oxide. A low dose of diazoxide (20 μg/paw) administered together with a low dose of sodium nitroprusside (125 μg/paw) or dibutyryl cGMP (db-cGMP, 50 μg/paw) induced a marked antinociceptive effect similar to that observed when each drug was administered alone. NS1619 (75, 150 and 300 μg/paw), a specific opener of large-conductance Ca^{2+}-activated K^+ channels, had no antinociceptive action on prostaglandin E_2-induced hyperalgesia. This series of experiments provides evidence for a peripheral antinociceptive action of diazoxide and supports the suggestion that the activation of ATP-sensitive K^+ channels could be the mechanism by which sodium nitroprusside and db-cGMP induce peripheral antinociception, excluding the involvement of large-contuctance Ca^{2+}-activated K^+ channels in the process.

Keywords: db-cGMP; Diazoxide; K^+ channel opener; Sodium nitroprusside; Antinociception

1. Introduction

It has been suggested that the molecular mechanism of the peripheral (Ferreira et al., 1991) and central (Duarte and Ferreira, 1992) antinociception induced by morphine involves activation of L-arginine/nitric oxide (NO)/cGMP. Morphine has been shown to exert its peripheral antinociceptive effect by activating ATP-sensitive K^+ channels (Rodrigues and Duarte, 2000). Recent studies from our laboratory demonstrated that the peripheral antinociceptive action of the NO donor, sodium nitroprusside (Soares et al., 2000), and of dibutyryl cGMP (db-cGMP; Soares and Duarte, 2001) is associated with ATP-sensitive K^+ channels, thus establishing a link between the participation of the NO/cGMP pathway in the analgesia induced by some drugs and the activation of ATP-sensitive K^+ channels.

These channels are controlled by the ATP:ADP ratio of the cell (DeWeille and Lazdunski, 1990). A high ATP:ADP ratio closes the channel and leads to cellular depolarization and neurotransmitter release. Conversely, opening of the

* Corresponding author. Tel./fax: +55-31-499-2695.
E-mail address: dimitri@mono.icb.ufmg.br (I.D.G. Duarte).

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doi:10.1016/j.ejphar.2004.02.022

channel leads to hyperpolarization and a decrease in neurotransmitter release. Diazoxide belongs to a class of drugs called ATP-sensitive K^+ channel openers due to their ability to enhance K^+ conductance via these channels (Newgreen et al., 1990). Oral hypoglycemic agents, such as glibenclamide, close ATP-sensitive K^+ channels (Edwards and Weston, 1993). In addition to the above effects, the central administration of K^+ channel openers, such as diazoxide, minoxidil, lemakalim and cromakalim, has been reported to produce antinociception in laboratory animals (Welch and Dunlow, 1993; Narita et al., 1993) and to potentiate the enhancement of the pain threshold produced by opioid and α_2-adrenoceptor agonists (Vergoni et al., 1992; Ocaña et al., 1996).

In the light of these facts, we undertook this project to answer the following questions: does the K^+ channel opener diazoxide produce peripheral antinociception and, if so, is this effect blocked by a specific K^+ channel blocker? How does the channel opener diazoxide interact with NO and db-cGMP-induced antinociception?

2. Materials and methods

2.1. Animals

The experiments were performed on 180–250 g male Wistar rats (from CEBIO-UFMG). The animals were housed in a temperature-controlled room (23 ± 1 °C) on an automatic 12-h light/dark cycle (6 a.m. to 6 p.m.) All tests were conducted during the light phase (12 a.m. to 5 p.m.). Food and water were freely available until the beginning of the experiments. Naive animals were used throughout.

2.2. Measurement of the hyperalgesia

Hyperalgesia was induced by subcutaneous injection of prostaglandin E_2 (2 μg) into the plantar surface of the hindpaw. The hyperalgesia was measured according to the paw pressure test described by Randall and Selitto (1957). An analgesimeter was used (Ugo-Basile, Italy) with a cone-shaped paw-presser with a rounded tip which applies a linearly increasing force to the hindpaw. The weight in grams required to elicit a nociceptive response such as paw flexion was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured in the right paw and determined as the average of the three consecutive trials recorded before and 3 h after prostaglandin E_2 injection. The threshold was calculated as the difference between these two averages (Δ of nociceptive threshold) and is expressed in grams.

2.3. Drug administration

The drug used as hyperalgesic agent was prostaglandin E_2 (Sigma). db-cGMP (N^2,2′-*O*-dibutyrylguanosine 3′: 5′ cyclic monophosphate, Sigma) and sodium nitroprusside (Sigma) were used as antinociceptive drugs. The K^+ channel openers were diazoxide (RBI) and NS 1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl) phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one, RBI). The K^+ channel blockers were glibenclamide (Sigma), tetraethylammonium chloride (Sigma), dequalinium chloride (Calbiochem) and charybdotoxin (Sigma). We used naloxone (Sigma) as an opioid receptor antagonist and methylene blue (RBI) as an inhibitor of guanylate cyclase. All drugs were dissolved in isotonic saline and injected in a volume of 100 μl per paw, except for diazoxide and glibenclamide, which were dissolved in 1% Tween, and NS-1619, which was dissolved in ethanol (PA) and 1% Tween at a proportion of 20% and 80%, respectively.

2.4. Experimental protocol

Sodium nitroprusside and db-cGMP were administered subcutaneously in the right hindpaw 2 h after prostaglandin E_2 injection. All the K^+ channel openers were injected subcutaneously into the right hindpaw 2 h and 45 min after prostaglandin E_2 administration. K^+ channel blockers were administered 5 min before diazoxide administration. Naloxone and methylene blue were injected 30 min before diazoxide. We assessed in pilot experiments the best moment for injection of each substance used, based on data in the literature (Ortiz et al., 2002). The nociceptive threshold was always measured in the right hindpaw.

2.5. Statistical analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) with post-hoc Bonferroni's test for multiple comparisons. Probabilities less than 5% ($P < 0.05$) were considered to be statistically significant.

3. Results

3.1. Hyperalgesic effect of prostaglandin E_2

Intraplantar administration of prostaglandin E_2 (0.1, 0.5 and 2 μg) produced a dose-dependent reduction of the nociceptive threshold to mechanical stimuli in rat paws when compared to the control threshold. The peak effect was observed 3 h after prostaglandin E_2 injection. Because the dose of 2 μg was able to induce marked hyperalgesia, it was used in the subsequent experiments (Fig. 1).

3.2. Antinociceptive action of diazoxide

The administration of diazoxide (20, 38, 75, 150, 300 and 600 μg) into the right hindpaw antagonized the hyperalgesic effect of prostaglandin E_2 (2 μg/paw), in a dose-dependent manner (Fig. 2). ED_{50} (222.9 μg) was calculated from a log dose–response curve (same figure, above). Diazoxide at the dose of 300 μg, when administered into

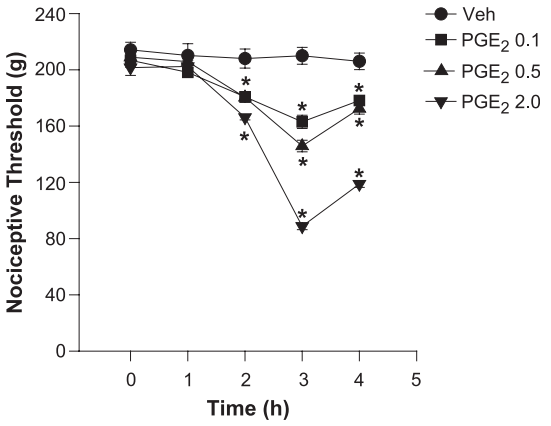

Fig. 1. Time course of the hyperalgesic effect induced by intraplantar injection of different doses (μg) of prostaglandin E_2 (PGE_2). Each point represents the mean ± S.E.M. ($n = 5$) of the nociceptive threshold expressed in grams (g). * Indicates a significant difference from the vehicle (8% ethanol in isotonic saline)-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

the left paw, did not produce an antinociceptive effect in the right paw, indicating that, at this dose, it had a peripheral site of action (Fig. 3).

3.3. Effect of different K^+ channel blockers on diazoxide-induced antinociception

As shown in Fig. 4, glibenclamide (40, 80 and 160 μg/paw) dose dependently reduced the diazoxide-induced peripheral antinociception (300 μg/paw), while tetraethylammonium (7.5, 15 and 30 μg), dequalinium (12.5, 25 and 50 μg) and charybdotoxin (0.5, 1 and 2 μg) injected into the paw failed to significantly counteract the antinociception induced by diazoxide (Fig. 5).

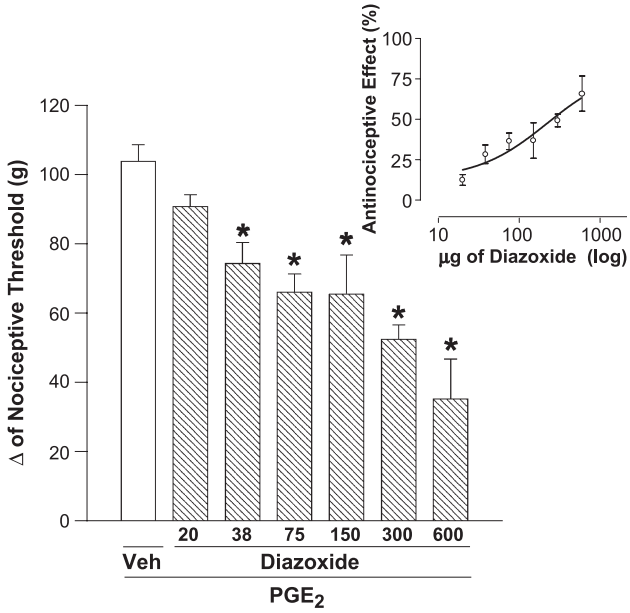

Fig. 2. Effect of diazoxide on the nociceptive threshold in rats with prostaglandin E_2-induced hyperalgesia. Diazoxide (μg/paw) was administered 2 h and 45 min after prostaglandin E_2 (PGE_2, 2 μg/paw).The antinociceptive response was measured in the paw-pressure test, as described in Methods. The insert shows a log dose–response curve ($ED_{50} = 222.9$ μg). Each column represents the mean ± S.E.M. ($n = 5$). * Indicates a significant difference from the PGE_2+ vehicle-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

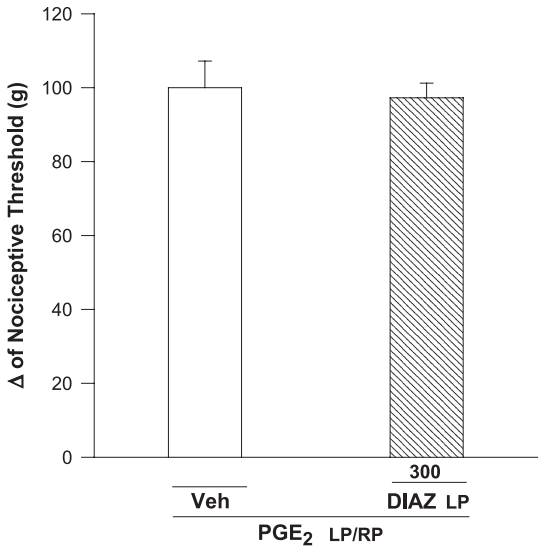

Fig. 3. Exclusion of an antinociceptive action of diazoxide (300 μg) at sites other than the paw. Diazoxide (DIAZ) was administered in the left paw (LP) 2 h and 45 min after prostaglandin E_2 administration in both hindpaws. The analgesic response of the right hindpaw (RP) was measured in the paw-pressure test described in Section 2. Each column represents the mean ± S.E.M. ($n = 4$). No statistically significant difference was found between the groups treated with PGE_2+ vehicle and PGE_2+ diazoxide LP.

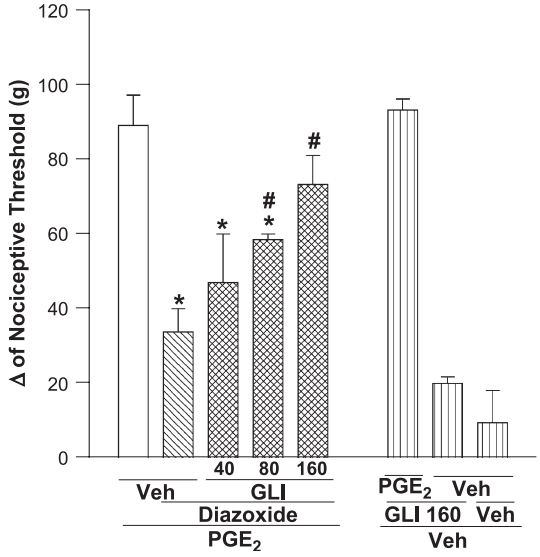

Fig. 4. Antagonism induced by intraplantar administration of glibenclamide of peripheral antinociception produced by diazoxide in hyperalgesic paws. Glibenclamide (GLI; 40, 80 and 160 μg/paw) was administered 5 min before diazoxide (300 μg/paw). Each column represents the mean ± S.E.M. ($n = 4$). * Indicates a significant difference from the PGE_2+ vehicle-injected control and # indicates a significant difference between PGE_2 + diazoxide + glibenclamide and PGE_2 + diazoxide + vehicle-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

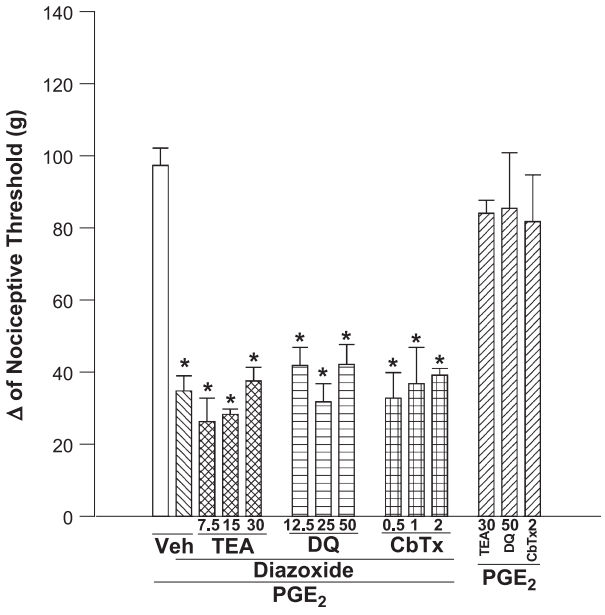

Fig. 5. Effect of intraplantar administration of tetraethylammonium (TEA; 7.5, 15 and 30 μg), dequalinium (DQ; 12.5, 25 and 50 μg) and charybdotoxin (CbTx; 0.5, 1 and 2 μg) on the peripheral antinociception induced by diazoxide in hyperalgesic paws. No statistically significant difference was found between the groups treated with PGE_2 + diazoxide + vehicle and PGE_2 + diazoxide + TEA or DQ or CbTx. * Indicates a significant difference from the PGE_2 + vehicle-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

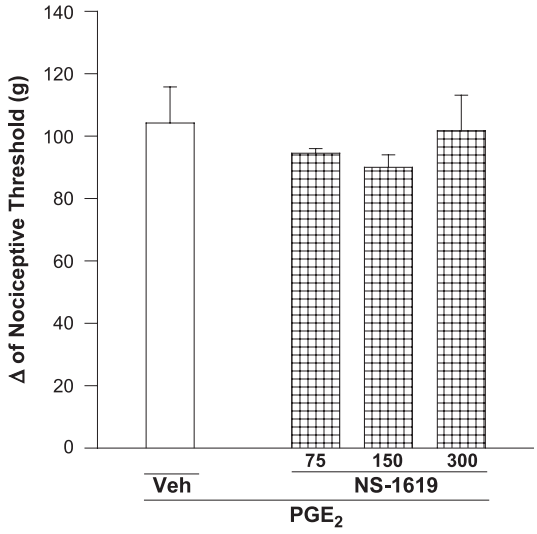

Fig. 7. Effect of NS-1619 in rats with prostaglandin E2-induced hyperalgesia (2 μg/paw). NS-1619 (75, 150 and 300 μg/paw) was administered 2 h and 45 min after PGE_2. The columns represents the mean ± S.E.M. ($n = 5$). No statistically significant difference was found between the groups treated with PGE_2 + vehicle and PGE_2 + NS-1619.

3.4. Effect of the combination of sodium nitroprusside or db-cGMP with diazoxide on prostaglandin E_2-induced hyperalgesia

Fig. 6A shows that sodium nitroprusside (125 μg/paw) and diazoxide (20 μg/paw) induced an antinociceptive effect of 21.3% and 29.3%, respectively, on prostaglandin E_2-induced hyperalgesia. The combination of sodium nitroprusside and diazoxide, at the same doses, produced a marked inhibition of prostaglandin E_2-induced hyperalgesia, of the order of 67.3%. The administration of a low dose of db-cGMP (50 μg/paw) in combination with a low dose of diazoxide (20 μg/paw) produced a significant reduction of prostaglandin E_2-induced hyperalgesia (2 μg/paw) of approximately 63.3%. When db-cGMP and diazoxide were administered alone, at the same doses, the observed inhibition of prostaglandin E_2-induced hyperalgesia was 38.0% and 23.3%, respectively (Fig. 6B).

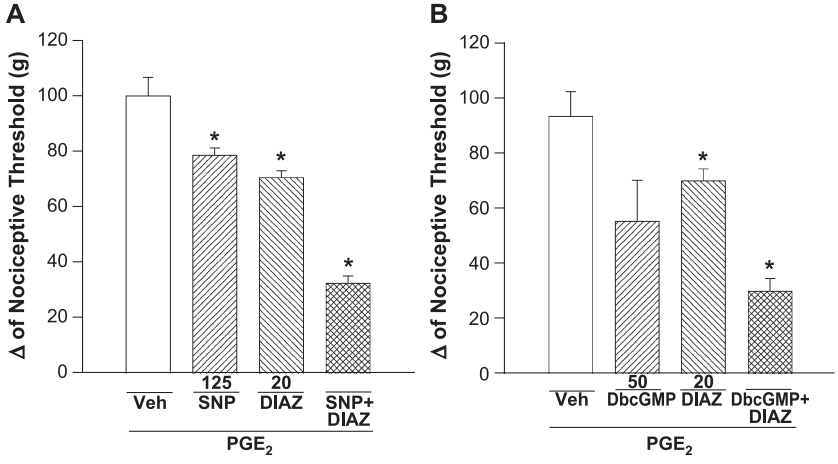

Fig. 6. (A) Effect of the combination of sodium nitroprusside (SNP) and diazoxide (DIAZ) on the hyperalgesic action of PGE_2 (2 μg/paw). (B) Effect of the combination of db-cGMP and diazoxide (DIAZ) on the hyperalgesic action of PGE_2 (2 μg/paw). SNP or db-cGMP μg/paw) was administered 2 h and diazoxide (μg/paw) 2 h and 45 min after PGE_2. The columns represent the mean ± S.E.M. ($n = 5$). * Indicates a significant difference from the PGE_2 + vehicle-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

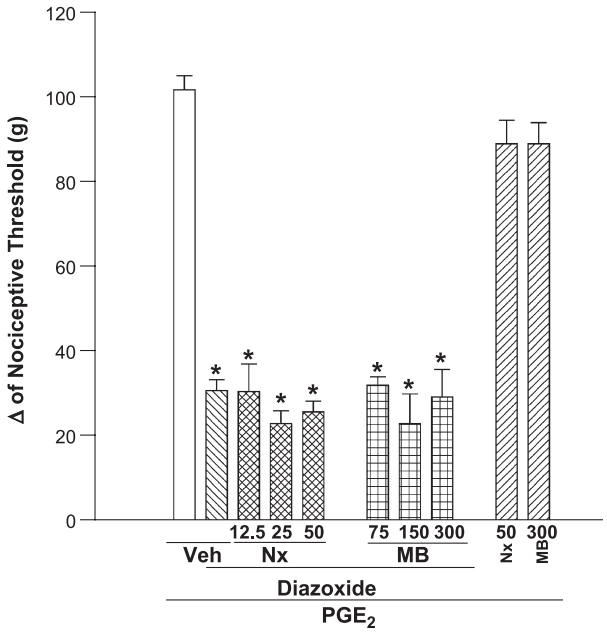

Fig. 8. Effect of intraplantar administration of naloxone (Nx, 12.5, 25 and 50 μg) or methylene blue (MB, 75, 150 and 300 μg) on the peripheral antinociception induced by diazoxide (300 μg/paw) in hyperalgesic paws (PGE_2, 2 μg). Nx or MB was administered 30 min before diazoxide. Each column represents the mean ± S.E.M. ($n = 5$). No statistically significant difference was found between the groups treated with PGE_2 + diazoxide + vehicle and PGE_2 + diazoxide + Nx or MB. * Iindicates a significant difference from the PGE_2 + vehicle-injected control ($P < 0.05$, ANOVA + Bonferroni's test).

3.5. Evaluation of the effect of NS 1619 on prostaglandin E_2-induced hyperalgesia

Fig. 7 shows that the intraplantar administration of different doses of NS 1619 (75, 150 and 300 μg), a direct opener of Ca^{2+}-activated K^+ channels, had no antinociceptive activity on prostaglandin E_2-induced hyperalgesia (2 μg/paw).

3.6. Absence of the involvement of an endogenous opioid system and of cGMP activation in diazoxide analgesia

Fig. 8 demontrates that neither naloxone (12.5, 25 and 50 μg/paw) nor methylene blue (75, 150 and 300 μg/paw) produced an effect on the peripheral antinociceptive action of diazoxide (300 μg/paw).

4. Discussion

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. The term "K^+ channel openers" was initially associated with a group of chemically diverse agents (such as cromakalim, pinacidil, nicorandil) that evoke K^+ efflux through ATP-sensitive K^+ channels. This finding initiated a search to identify molecules that specifically open other K^+ channel subtypes (Lawson, 2000).

Many studies have demonstrated an antinociceptive effect associated with ATP-sensitive K^+ channel activation. Vergoni et al. (1992) demonstrated that pinacidil increased and prolonged the central antinociceptive effect of morphine measured in the hot-plate and tail-flick tests. In 1993, Welch and Dunlow, also using the tail-flick test, observed an antinociceptive effect induced by intrathecal administration of ATP-sensitive K^+ channel openers such as diazoxide, minoxidil and lemakalim. Ocaña and Baeyens (1994), using the tail-flick test, demonstrated that intracerebroventricularly administered cromakalim increased in a dose-dependent manner the antinociceptive effect of the adenosine A_1 receptor agonist (R-PIA). Similarly, Ocaña et al. (1995) and Robles et al. (1996), using the tail-flick test and the hot-plate test, respectively, demonstrated that cromakalim administered by the intracerebroventricular route potentiated in a dose-dependent manner the central antinociceptive effect induced by morphine and by agonists of the $5\text{-}HT_{1A}$ receptor. Shewade and Ramaswamy (1995) also demonstrated that minoxidil, another ATP-sensitive K^+ channel activator, administered intraperitoneally, potentiated the antinociceptive action of prolactin, measured in the abdominal writhing test. More recently, Galeotti et al. (2001) demonstrated that the central antinociception induced by amitriptyline and clomipramine, tricyclic antidepressants, assessed in the hot-plate test in mice, was prevented by the ATP-sensitive K^+ channel blocker gliquidone and potentiated by two activators of these channels, minoxidil and pinacidil.

In our experiments, diazoxide had a dose-dependent peripheral antinociceptive effect on the hyperalgesia induced by prostaglandin E_2. In order to exclude the possibility that diazoxide at the dose of 300 μg/paw produced analgesia by acting at sites other than the paw, we used the strategy of evaluating the efficacy of ipsilateral versus contralateral paw administration. We chose this strategy because the route and site of injection would be the same. Prostaglandin E_2 was administered into both hind paws, thus creating the same tissue conditions and an equal possibility that the agents tested would reach sites outside the injected paw. Taking into account that the nociceptive threshold was always measured in the right hind paw, diazoxide at the dose of 300 μg was ineffective when administered into the contralateral paw, suggesting that at this dose diazoxide has a peripheral site of action. This is the first time that a peripheral antinociceptive action of a K^+ channel opener has been demonstrated, confirming the existence and function of these channels in nociception.

Our results demonstrated that the sulfonylurea glibenclamide could prevent in a dose-dependent manner the peripheral antinociceptive effect induced by diazoxide.

Sensitivity to sulfonylureas is commonly used to characterize ATP-sensitive K^+ channels (Babenko et al., 1998). Glibenclamide did not cause any hyperalgesic or antinociceptive effect when administered alone, suggesting that these channels are not tonically activated in primary afferent neurons. In contrast, we tested different doses of charybdotoxin, a toxin that blocks large-conductance Ca^{2+}-activated K^+ channels (Miller et al., 1985), dequalinium, a selective blocker of small-conductance Ca^{2+}-activated K^+ channels (Dunn, 1994), and tetraethylammonium, a non-selective voltage-dependent K^+ channel blocker (Cook and Quast, 1990), and observed that all these drugs failed to antagonize the peripheral antinociception induced by diazoxide. None of the K^+ channel blockers studied was able to induce an antinociceptive effect on prostaglandin E_2-induced hyperalgesia.

It is interesting to note that analgesics such as morphine (Rodrigues and Duarte, 2000) and dipyrone (Alves and Duarte, 2002) act peripherally by activating ATP-sensitive K^+ channels. In addition, diazoxide in combination with low doses of sodium nitroprusside or dibutyryl cGMP (Soares et al., 2000; Soares and Duarte, 2001) can strongly potentiate the antinociceptive effect of both drugs. Thus, the above results provide evidence in favor of the involvement of ATP-sensitive K^+ channels in the antinociceptive action of several substances, including sodium nitroprusside and db-cGMP.

Among the different and numerous K^+ channel families, medicinal chemistry has mainly focused on two channel types: the ATP-sensitive channels and the large-conductance subtype, which belongs to the broad family of Ca^{2+}-activated K^+ channels. Large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) channels have been investigated as potential therapeutic targets for different neuropathies because of their profound influence on neuronal activity. Morover, BK_{Ca} channels are expected to have applications for the therapy of cardiovascular diseases (Calderone, 2002). In order to investigate the participation of this type of K^+ channel in antinociception, we used different doses of NS-1619, a selective BK_{Ca} channel activator (Olesen et al., 1994). This activator did not show an antinociceptive action on prostaglandin E_2-induced hyperalgesia, suggesting that these channels do not exist in peripheral tissues or, more probably, that they are not activated under our experimental conditions.

It has been reported that ATP-sensitive K^+ channel openers produce antinociception that is attenuated by opioid receptors antisenses and antagonists (Lohmann and Welch, 1999; Campbell and Welch, 2001), indicating that ATP-K^+ channel openers produce antinociception, in part, via the release of endogenous opioid peptides. In the experimental model used, we did not obtain evidence supporting this hypothesis since the administration of naloxone, an opioid receptor antagonist, at doses able to reverse the peripheral antinociceptive effect of morphine (unpublished data) did not antagonize the antinociception induced by diazoxide. We also observed that the antinociceptive effect of diazoxide did not seem to be related to the increase in cGMP levels since different doses of methylene blue, a guanylate cyclase inhibitor, did not change this effect. Newgreen et al. (1990), in a study of the action of diazoxide and minoxidil on the blood vessels of rats, also observed that the effect of diazoxide did not involve cAMP or cGMP production.

This series of experiments provides evidence for a peripheral antinociceptive action of diazoxide and supports the suggestion that the activation of ATP-sensitive K^+ channels could be the mechanism by which sodium nitroprusside and db-cGMP induce peripheral antinociception, excluding the involvement of large-contuctance Ca^{2+}-activated K^+ channels in the process.

Acknowledgements

Research supported by Conselho Nacional de Pesquisa (CNPq, grants 000521719/95) and Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG, CBS-1800/95).

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