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Opposite nociceptive effects of the arginine/NO/cGMP pathway stimulation in dermal and subcutaneous tissues

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1 Nitric oxide has been described either as pronociceptive or antinociceptive. In this investigation, using an electronic pressure-metre, the intradermal and the subcutaneous effects of prostaglandin E_2 (PGE_2) and agents that mimic or inhibit the arginine/NO/cGMP pathway were compared.

2 The hypernociceptive effect of the intradermal injection of PGE_2 (100 ng) was immediate, peaking within 15–30 min and returning to basal values in 45–60 min. The subcutaneous injection of PGE_2 induced a hypernociception with a delayed peak (3 h) plateauing for 4–6 h.

3 Intradermal administration of 3-morpholino-sydnonimine-hydrochloride (SIN-1) enhanced, while its subcutaneous administration inhibited, subcutaneous hypernociception induced by PGE_2. This inhibition was prevented by ODQ (8 μg) but not by NG-monomethyl-L-arginine (L-NMMA) (50 μg).

4 Intradermal but not subcutaneous administration of L-arginine (1–100 μg), SIN-1 (1–100 μg) and dibutyrylguanosine 3′:5′-cyclic monophosphate (db cGMP) (0.1–100 μg) induced an early (15–30 min) dose-dependent hypernociceptive effect. Intradermal pretreatment with NG-monomethyl-L-arginine (L-NMMA; 50 μg) inhibited the hypernociception induced by L-Arg (10 μg), but not that induced by SIN-1 (10 μg) or db cGMP (10 μg).

5 Intradermal injection of ODQ (8 μg) antagonized the hypernociception induced by L-arginine and SIN-1, but not that induced by db cGMP.

6 Considering (a) the different time course of intradermal and subcutaneous PGE_2-induced hypernociception, (b) the opposite nociceptive effect of intradermal and subcutaneous administration of SIN-1 (db cGMP) as well as the arginine/NO/cGMP pathway, the existence of different subsets of nociceptive primary sensory neurons in which the arginine/NO/cGMP pathway plays opposing roles is suggested. This hypothesis would explain the apparent contradictory observations described in the literature.

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Abbreviations: db CAMP, 2′-*O*-dibutyrylguanosine 3′:5′-cyclic monophosphate; db cGMP, dibutyrylguanosine 3′:5′-cyclic monophosphate; L-NMMA, NG-monomethy-L-arginine; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one; PGE_2, prostaglandin E_2; SIN-1, 3-morpholino-sydnonimine-hydrochloride

Introduction

During the inflammatory response there is a functional upregulation of nociceptors, which leads to a clinical state known as hyperalgesia or allodynia, common denominator of inflammatory pain. The characteristic sensation of inflammatory pain provoked by mild mechanical stimulation, in general, cannot be reproduced in normal tissues even by an intense strong mechanical stimulation. In recent years, a special type of nociceptor 'switched on' during inflammation was described. This 'sleeping' or 'silent' nociceptor is associated with certain small afferent fibres in deep visceral enervation (Habler *et al.*, 1988; McMahon & Koltzenburg, 1990a, b) joints (Schaible & Schmidt 1985; 1988) and in skin (Kress *et al.*, 1992; Schmidt *et al.*, 1995). Prostaglandins sensitize these nociceptors (McMahon & Koltzenburg, 1990b). The involvement of this subset of peripheral nociceptors may be crucial for the development of mechanical hyperalgesia/allodynia in man and sensitization of nociceptor (hypernociception) in experimental animals.

Prostaglandins were shown to produce long-lasting skin hyperalgesia and their joint infusion with noneffective concentration of putative pain substances (bradykinin and histamine) produced overt pain (Ferreira, 1972). Those observations together with the fact that the synthesis of prostaglandins were inhibited by aspirin-like drugs (Ferreira *et al.*, 1971; Smith & Willis, 1971; Vane, 1971) supported our suggestion that the mechanism of action of nonsteroidal anti-inflammatory drugs resulted from the prevention of the sensitization of pain receptors (Ferreira, 1972). Eicosanoid-induced hypernociception became a paradigm of the final step in the cascade of hyperalgesic mediators, which occurs during the inflammatory response. We and other investigators, using different variations of the Randall–Selitto mechanical test,

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observed a late (2–3 h) induction of prostaglandin E_2 (PGE_2)-induced paw sensitization by subcutaneous (intraplantar) injection of PGE_2 (Ferreira *et al.*, 1978a, b; Picolo *et al.*, 2000; Safieh-Garabedian *et al.*, 2000). In contrast, Khasar *et al.* (1993), using the classical Randall–Selitto (paw pressure) model, reported that intradermal but not subcutaneous injection of PGE_2 and bradykinin caused an early (30 min) hyperalgesia. Another difference was observed with intradermal and subcutaneous injections of L-NMMA, L-Arg and an NO donor (SIN-1). These agents caused an early hyperalgesic effect (Aley *et al.*, 1998), but had no effect when given subcutaneously to normal paws (Duarte *et al.*, 1990). Bradykinin-induced hypernociception after intradermal injections was described to involve the arginine/NO/cGMP pathway (Nakamura *et al.*, 1996). On the other hand, with experiments using subcutaneous administration we showed that the arginine/NO/cGMP pathway counteracted ongoing inflammatory hypernociception (Duarte *et al.*, 1990; Cunha *et al.*, 1999).

These discrepancies prompted us to carry out experiments comparing the effects of subcutaneous and intradermal injections of PGE_2 and of agents that either mimic the activation or inhibit the arginine/NO/cGMP pathway. In this study, an electronic pressure-metre was used to quantify mechanical hypernociception in rat paws (an electronic version of the von Frey hair test).

Methods

Animals

The experiments were performed on 180–200 g male Wistar rats (University of São Paulo, Brazil) housed in an animal care facility and taken to the testing area at least 1 h before the experiment. Food and water were available *ad libitum*. All behavioural testing was performed between 09.00 and 16.00 h. Rats were used once only. Animal care and handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines on the use of animals in pain research. All efforts were made to minimize the number of animals used and any discomfort.

Nociceptive paw electronic pressure-metre test for rats

In a quiet room, rats were placed in acrylic cages (12×20 by 17 cm high) with a wire grid floor, 15–30 min before testing. During this adaptation period, the paws were tested 2–3 times. The test consisted of provoking a hindpaw flexion reflex with a hand-held force transducer with a 0.5 mm^2 polypropylene tip (electronic von Frey hair, TC Inc. Life Science Instruments, U.S.A.). A tilted mirror below the grid provided a clear view of the rat hindpaw. The investigator was trained to apply the tip between the five distal footpads with a gradual increase in pressure. The maximal applied force was 80 g. The stimulus was repeated (up to six times, but usually, three times) until the animal presented two similar measurements. The end point was the paw withdrawal followed by a clear flinch response. The animal was discarded if it did not present a consistent response. The measurement of the pressure (calibrated in grams) was automatically recorded when the paw was withdrawn. As only few animals were discarded. The results, intensity of hypernociception, were presented as Δ withdraw threshold. The intensity of hypernociception was obtained by subtracting values measured at the defined time interval from those obtained in the control period, that is, before the injections.

Drugs

N^2, 2′-*O*-dibutyrylguanosine 3′:5′-cyclic monophosphate (db cGMP), N^6, 2′-*O*-dibutyrylguanosine 3′:5′-cyclic monophosphate (db cAMP), L-arginine (L-Arg) and prostaglandin E_2 (PGE_2) were purchased from Sigma (St Louis, MO, U.S.A.). NG-monomethyl-L-arginine (L-NMMA) and 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were purchased from Tocris Cookson Inc. (St Louis, MO, U.S.A.). 3-Morpholinosydnonimine-hydrochloride (SIN-1) was obtained from Pharmaforschung Galenik (Frankfurt, Germany).

L-Arginine, Db cGMP, L-NMMA and SIN-1 were diluted in sterile saline. A stock solution of PGE_2 was prepared in 10% ethanol, and further dilutions were made in saline; the final concentration of ethanol was less than 1%. ODQ was dissolved in DSMO (1 $\mu g\,\mu l^{-1}$) and diluted in saline.

Intradermal and subcutaneous injections

Drugs were injected into the rat hindpaw either intradermally or subcutaneously. A hypodermic 26 g needle was inserted into the skin of the second footpad and the injection was given between the five distal footpads at the same place where mechanical stimulus was applied. A volume of 20 μl was injected when intradermal and subcutaneous injections were compared. When only subcutaneous injections were used, a volume of 50 μl was administered. For injections, rats were anaesthetized with ether (Chemo, Indústria e comércio LTDA, Campinas, SP, Brazil), recovering within 3 min of the discontinuation of the anaesthesia. After this short anaesthesia, they did not present overt signs of stress or broncosecretion.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to compare the effects of different doses or treatments followed by the Tukey test. Results with $P<0.05$ were considered statistically significant.

Results

Differential time course of intradermal and subcutaneous PGE_2 induced a mechanical hypernociception

Intradermal PGE_2 (100 ng) administration induced a mechanical hypernociception within 15–30 min returning after 45 min to the level of saline injection (Figure 1). On the other hand, hypernociception induced by subcutaneous injection of PGE_2 (100 ng) commenced after 1 h, reaching a peak 2–3 h after the treatment and persisting for a further 2–3 h (not shown data). The results of intradermal and subcutaneous injections of saline were pooled since there were no conspicuous differences among them. Saline produced no significant change in the paw sensitivity, as shown by the dashed line (Figure 1).

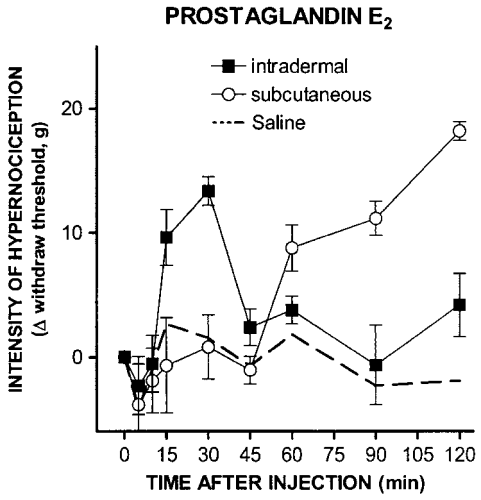

Figure 1 Time course of mechanical hypernociception induced by intradermal and subcutaneous injections of PGE_2 (100 ng/paw). The saline curve represents the pooled data of intradermal- and subcutaneous saline- (20 μl) injected groups. The data are reported as mean $\pm$s.e.m. of 5–6 animals per group.

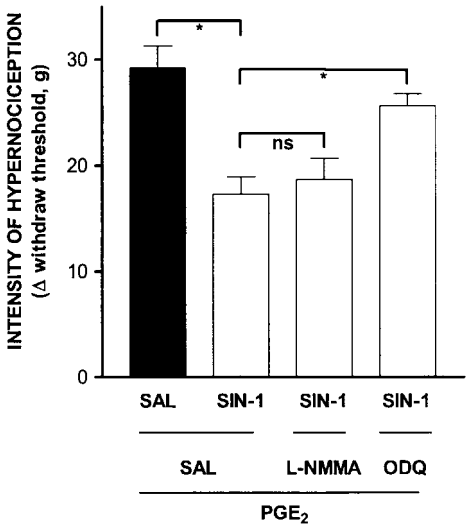

Figure 2 Effect of L-NMMA and ODQ on the antinociceptive effect of administration of SIN-1 on the hypernociceptive effect induced by injection of PGE2 (100 ng). All agents were injected subcutaneously. L-NMMA (50 μg), ODQ (8 μg) or saline (50 μl) were given 1.5 h and SIN-1 (200 μg) 2 h after PGE_2 administration. Measurements were made 3 h after PGE_2 injection. The results are expressed as the mean $\pm$s.e.m. of 5–6 animals per group. Astrisk (*) and NS indicate significant and no significant difference, respectively.

Effect of subcutaneous L-NMMA and ODQ on the antinociception induced by subcutaneous administration of SIN-1

The full bar of Figure 2 shows the control hypernociception measured 3 h after the subcutaneous administration of PGE_2 (50 ng). The subcutaneous administration of SIN-1 (200 μg), 1.5 h before the measurements, significantly reduced PGE_2 mechanical hypernociception. Pretreatment of the paws with ODQ (8 μg) 30 min before subcutaneous administration of SIN-1 significantly reversed its antinociceptive effect. L-NMMA (50 μg) did not affect the antinociceptive effect of SIN-1 (Figure 2).

Opposite effects of intradermal and subcutaneously injections of SIN-1 on PGE_2 subcutaneously by induced hypernociception

The full bars of Figure 3 are the control hypernociceptive effects (different animal groups) measured 3 (a) and 4 (b) h after subcutaneous injections of PGE_2 (50 ng). Panel a shows that intradermal injection of SIN-1 (1 μg), given 15 min before the measurements, similarly to that shown in panel c of Figure 4, caused hypernociception. There were no significant differences when PGE_2 and SIN-1 effects were compared. However, when both treatments were made, the hypernociception was significantly greater than that induced by PGE_2 only. SIN-1 given subcutaneously (100 μg), 1 h before the measurement, had no effect *per se* (similar to that saline, not shown) but significantly inhibited PGE_2-induced hypernociception.

Differential hypernociception induced by intradermal and subcutaneous injections of L-arginine, SIN-1 and db cGMP

The left panels of Figure 4 show the time course of intradermal (full dots) and subcutaneous (open dots) injections of L-arginine (a), SIN-1 (b) and db cGMP (c). The intradermal doses of the agents were supramaximal (100 μg) as shown in the lower panel. The lower panels show the dose response induced by L-arginine (d), SIN-1 (e) and db cGMP (d) measured 15 min after injection. No effect was observed 3–4 h after injection (data not shown).

The curves for the intradermal injections (panels a–c) were significantly different from those for subcutaneous injections (ANOVA). The curves for subcutaneous injections did not differ from saline curves (ANOVA), but there was a tendency to cause hypernociception, which may result from the diffusion of the drugs to intradermal tissues. There was no difference in the responses to intradermal and subcutaneous saline injections (data not shown, but similar time course of saline injections is shown in panel a of Figure 5).

Differential inhibitory effects of L-NMMA and ODQ on paw mechanical hypernociception induced by intradermal injection of L-arginine, SIN-1 and db cGMP

Panel a of Figure 5 shows that the intradermal effect of L-NMMA (50 μg) and ODQ (8 μg) does not differ from saline (20 μl). Arginine-induced intradermal hypernociception was inhibited by both L-NMMA and ODQ (panel b). SIN-1 intradermal-induced hypernociception (panel c) was antagonized by ODQ but not by L-NMMA pretreatments that had no effect upon db cGMP-induced nociceptor sensitization (panel d).

Time course effects of intradermal and subcutaneous injections of db cAMP induced hypernociception

Figure 6 shows the time course of the nociception induced by the same dose of db cAMP (100 μg) given by the intradermal

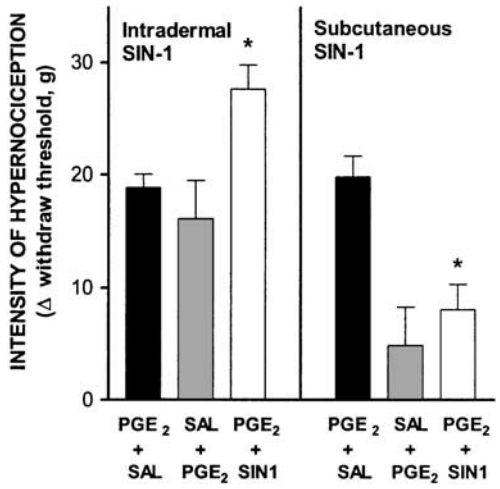

Figure 3 Opposite effects of intradermal (a) and subcutaneous (b) injections of SIN-1 on subcutaneous PGE_2-induced mechanical hypernociception. The measurements were made 3 (a) or 4 h (b) after the intraplantar injection of PGE_2 (50 ng). SIN-1 was injected intradermally (1 μg/paw) 15 min or subcutaneously (100 μg/paw) 1 h before the measurements. The results are reported as mean $\pm$s.e.m. of 5–6 animals per group. *Indicate significant difference compared to PGE_2 group.

and subcutaneous routes. The intradermal and subcutaneous hypernociceptive effect reached a peak within 15–30 min after the injection. The intradermal effect subsided within 60–90 min, while the subcutaneous effect remained for more than 4 h.

Discussion

In animal experiments the term nociception is generally used for behaviours similar to human pain. Hyperalgesia and allodynia were terms originally used to describe different types of human pain and extended to animal experiments based upon the fact that the noxious or non-noxious stimulation of normal tissue may or may not evoke a behavioural end point. The von Frey test is described in the literature as an allodynic test (Kim & Chung, 1992; Sousa & Prado, 2001). Since it is rather questionable to conclude that these animal tests measure human symptoms corresponding either to allodynia or hyperalgesia, in the present investigation we used the term hypernociception to denote a decrease of the intensity of mechanical stimulation capable of producing a standardized animal behaviour (hypernociceptive behaviour). As accepted for allodynia and hyperalgesia, we assumed that hypernociception reflects an increased sensitivity of nociceptors. The use of the term hypersensitivity and hyperexcitability was avoided because they have a precise meaning in immunological and electrophysiological experiments, respectively. In recent years, classical von Frey filaments became popular for mechanical tests using rats (Kim & Chung, 1992; Omote *et al.*, 2001; Sousa & Prado, 2001). In the present investigation, a commercial electronic pressure-metre similar to an instrument used to successfully quantify neuropathic allodynia in rats was used to quantify mechanical hypernociception (Möller *et al.*, 1998). Although there is an apparent similarity to the classical von Frey test, the electronic pressure-metre uses a single stimulation of increasing pressure instead of sequential applications with various filaments of variable stiffness. In pilot experiments, it was observed that in naive rat hindpaws, the hardest von Frey filament or the electronic pressure-metre may or may not induce the behavioural end point used in this work (removal of the paw followed by clear flinches). In general, the failure to induce the end point was much higher with the von Frey filaments than with the electronic pressure metre. In the presence of induced hypernociception, there was a correlation between both tests.

It was described here that the nociceptive effect of intradermal-injected PGE_2 was rapid, reaching a peak within 15–30 min and returning to basal values within 45 min. Subcutaneous injection of PGE_2 within this time frame had little or no effect. These results confirm the observations of Khasar *et al.* (1993) using the classical Randall–Selitto pressure test. We also described that hypernociception induced by subcutaneous injection of PGE_2 began 45–60 min after its administration, reached a plateau after 3 h and persisted for a further 2–3 h. A similar pattern of nociception occurs in both the classical and in our modified Randall–Selitto test (Ferreira *et al.*, 1978b, Soares & Duarte, 2001). Khasar *et al.* (1993) failed to observe the subcutaneous hypernociceptive effect of PGE_2 because they limited their observation period to 1 h.

In this study, it was also shown that intradermal administration of L-arginine and SIN-1 induced an early (15–30 min) dose-dependent hypernociception. These results confirm the observations of Aley *et al.* (1998) using the classic Randall–Selitto test. We also confirmed an early study made with a different mechanical test and subcutaneous administration, that SIN-1 and db cGMP cause antinociception, i.e. blocked the hypernociceptive effect of PGE_2 (Duarte *et al.*, 1990). Illustrating the differential sensitivities of dermal and subcutaneous nociceptors, we showed that SIN-1 given intradermally enhanced the hypernociception induced by subcutaneous injected PGE_2. We also confirmed that subcutaneous SIN-1 seems to cause antinociception by the stimulation of guanylate cyclase since its effect was blocked by ODQ (Duarte *et al.*, 1992; Cunha *et al.*, 1999). The present investigation (Figures 2 and 3) supports that the arginine/NO/cGMP pathway is antinociceptive in subcutaneous tissues while it is pronociceptive in intradermal tissues. Indeed, it was shown that the intradermal pretreatment with L-NMMA blocked the hypernociceptive effect of L-arginine but not that induced by SIN-1 or db cGMP. These results confirm observations of Aley *et al.* (1998) made with the mechanical Randall–Sellito test concluding that intradermal NO produces a cGMP-dependent hyperalgesia.

There was another clear difference between the two routes of administration since the effect of a supramaximal intradermal dose of db cAMP was of much shorter duration than that observed with its subcutaneous administration (Figure 6). It is well known that the antiangina NO releaser, nitroglycerin, causes headaches (Kerins *et al.*, 2001). NO evoked pain when injected intracutaneous in humans (Holthusen & Arndt, 1994). On the other hand, there are opposite observations in man that drugs which release NO *per se* have no effect but are analgaesic or enhance the effect of other analgaesics when tested in models of ongoing pain or hypernociception in humans or in rats. We have proposed that the peripheral analgaesic effect of morphine (Ferreira *et al.*, 1991), dipyrone (Lorenzetti & Ferreira, 1985) and diclofenac (Tonussi & Ferreira, 1994) was associated with the stimulation of the arginine/NO/cGMP pathway. Recently, a family of NSAID containing NO in the

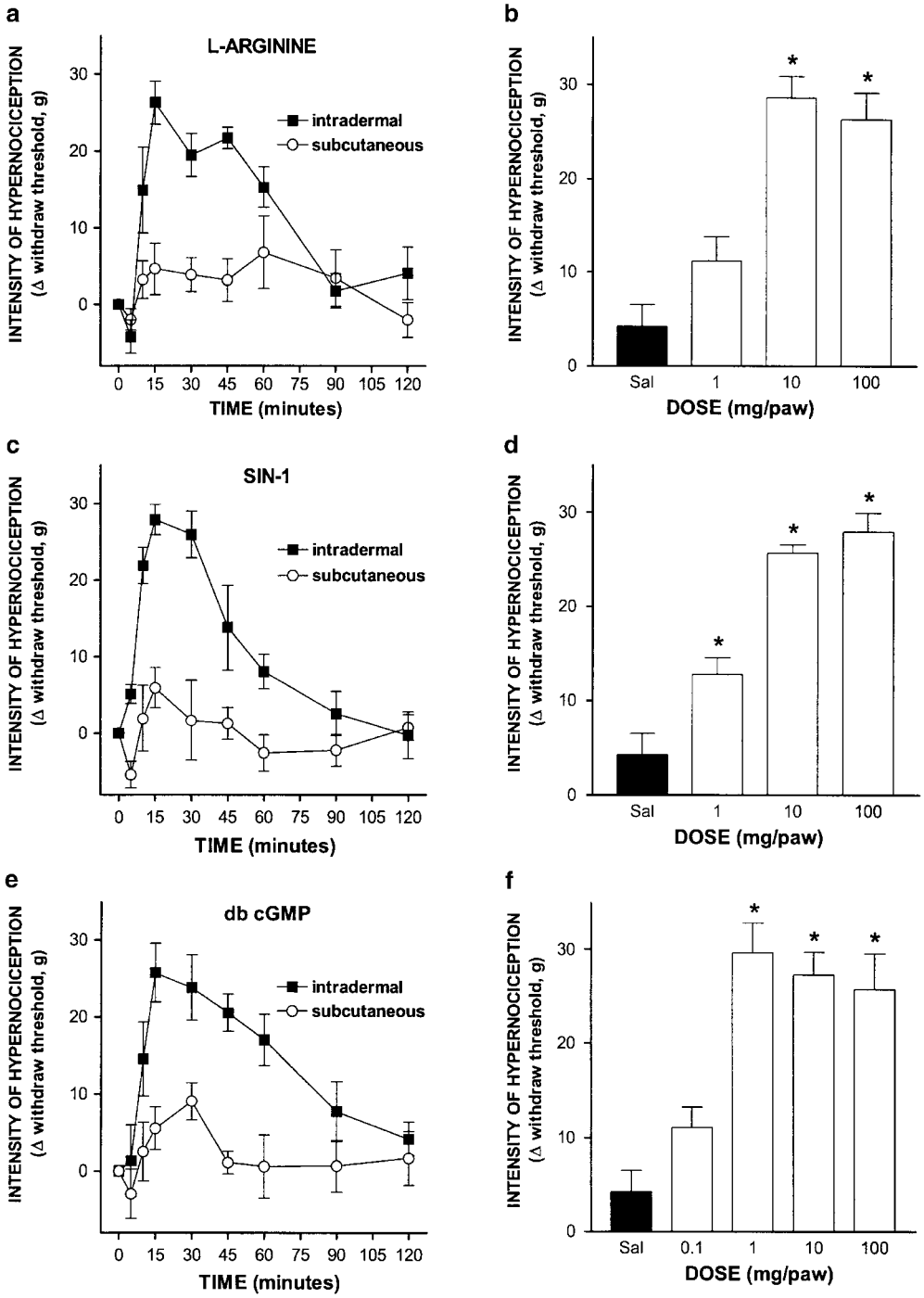

Figure 4 The left panels show the time course after intradermal and subcutaneous injections of L-arginine (a, 100 μg), SIN-1 (c, 100 μg) and db cGMP (e, 100 μg). The right panels (b, d and f) show the dose-dependent effects measured 15 min after the administration of L-arginine, SIN-1 db and cGMP, respectively. The results are the mean $\pm$s.e.m. of 5–6 animals per group.

molecule have been shown to be significantly more antinociceptive than the parent compound (Cicala *et al.*, 2000). We have no explanation why NO causes peripheral nociception. The peripheral effect of substances which stimulate is becoming clearer, since it has been demonstrated in hypernociceptive models, that the peripheral antinociceptive effect of NO donors, db cGMP, morphine and dipyrone is because of the opening of ATP-dependent K^+ channels. This promotes the K^+ outward currents, which may counteract the lowering of the nociceptor threshold (Rodrigues & Duarte, 2000; Soares *et al.*, 2000). It is thought that PGE_2 causes intradermal (Taiwo *et al.*, 1989; Hingtgen *et al.*, 1995) and subcutaneous hypernociception with the involvement of the cAMP/Ca2+/PKA pathway (Ferreira & Nakamura, 1979; Cunha *et al.*, 1999). Hypernociception caused by PKA may result from the lowering of the nociceptor threshold because of Ca mobilization and closure of K^+ channels (Evans *et al.*, 1999).

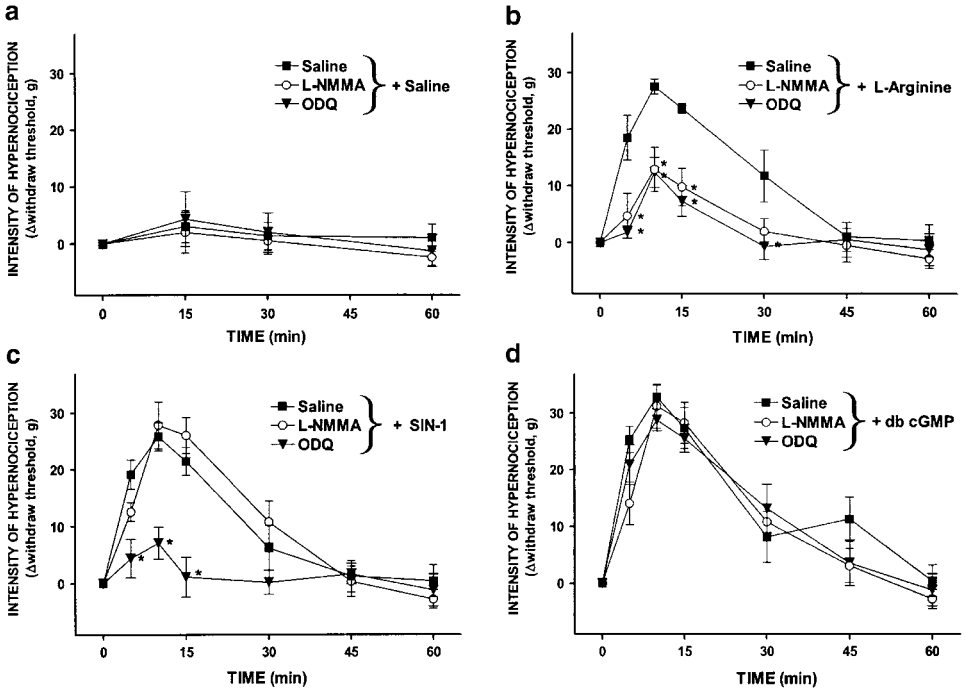

Figure 5 Effect of L-NMMA and ODQ on the paw mechanical hypernociception caused by intradermal injection of L-arginine (10 μg), SIN-1 (10 μg) and db cGMP (10 μg). Intradermal pretreatment with L-NMMA (50 μg) and ODQ (8 μg) was performed 30 min before administration of the hypernociceptive agents and the measurements were made 15 min after the intradermal challenge. The results are reported as the mean $\pm$s.e.m of 5–6 animals per group. *Indicates significant difference compared to saline pretreatment.

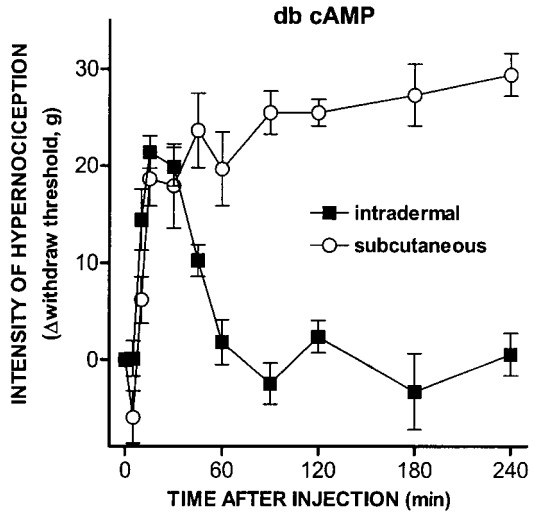

Figure 6 Differential time course of the intradermal and subcutaneous injections of db cAMP (100 μg). Results are reported as the mean $\pm$s.e.m. of 5–6 animals per group.

The simplest explanation for the discrepancies found in the literature may lay in the differential effect that NO has in the intradermal and subcutaneous nociceptors. The differences between the intradermal and subcutaneous nociceptors may result from the fact that both tissues are predominantly innervated by different subsets of primary nociceptive neurons. The presence of different subsets of C fibres has already been noted in spinal cord slices. In this preparation, the application of 8-br cGMP caused an excitation of every neuron that was excited by a NO donor and inhibited every cell that was inhibited by the NO donor (Pehl & Schmidt, 1997). In line with two different sets of neuronal pathways was the observation that inhibition or stimulation of mechanical hypernociception was observed following intrathecal injection of a NO donor (SIN-1) at small and large doses, respectively (Sousa & Prado, 2001). Kawabata *et al.* (1994) also suggested that peripheral NO plays a dual role in nociceptive modulation in the formalin test. These results may reflect a differential diffusion of the increasing concentrations of agents to subsets of primary sensory nociceptive neurons projecting to different laminae in the posterior dorsal horn.

In nociceptive tests using thermal stimulation, dermal nociceptors might be more readily stimulated than subcutaneous nociceptors. In thermal tests, the cGMP pathway mainly plays a hypersensitizing role (Meller *et al.*, 1992a, b) similar to that of the cAMP pathway. With chemical stimulation, such as in the formalin test, the stimulus may diffuse to both subcutaneous and cutaneous tissues, making the effect of pretreatment with modulators of the cGMP pathway hard to predict.

In summary, the results presented here support the suggestion that the arginine/NO/cGMP pathway plays opposing nociceptive roles in dermal and subcutaneous tissues, while cAMP (or stimulation of its formation) causes hypernociception in both sites. Our results emphasize the need to take into account, when comparing different sets of results, the site of the injection as well as the timing of the observation after pharmacological treatments.

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