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Endogenous opioids suppress activation of nociceptors by sub-nanomolar nicotine

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1 Nicotine can activate primary afferent nociceptors, one result of which is to increase neurogenic plasma extravasation. In this study we have demonstrated a novel proinflammatory effect of sub-nanomolar nicotine, mediated by peripheral action at sensory neurons. This action is normally masked by adrenal medulla-derived δ-opioid receptor agonists.

2 While neurogenic plasma extravasation in the knee joint of the rat was not increased by intra-articular perfusion of nicotine (10^{-8} M), perfusion of nicotine, at concentrations as low as 10^{-10} M, combined with naloxone to block opioid receptors (or naltrindole to block δ-opioid receptors) was able to enhance bradykinin-induced plasma extravasation. This pro-inflammatory effect of intra-articular nicotine was mimicked by subcutaneous nicotine which was abolished by intra-articularly-administered hexamethonium, a nicotinic receptor antagonist.

3 Following denervation of the adrenal medulla, intra-articular nicotine, alone at 10^{-8} M, enhanced plasma extravasation, which was no longer enhanced by naloxone.

4 Destruction of primary afferents by neonatal treatment with capsaicin or blockade of sensory neurotransmitter by neurokinin-1 receptor antagonist RP-87,580 abolished the pro-inflammatory effect of nicotine.

5 The effect of nicotine we describe in promoting inflammation is exerted at extremely low concentrations and therefore could have relevance to smokers, patients receiving medicinal nicotine as therapy and even second-hand smokers. Since receptor mechanisms on peripheral terminals of nociceptors may also be present on central terminals, actions of the endogenous nicotinic agonist acetylcholine, at central terminals of primary afferents or at other sites in the central nervous system, may be similarly modulated by opioids.

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Abbreviations: ANOVA, analysis of variance; BK, bradykinin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide; EDTA, ethylenediaminetetraacetate; EtOH, ethanol; NGF, nerve growth factor; PE, plasma extravasation

Introduction

Peripherally administered nicotine activates nociceptors (Sucher *et al*., 1990; Steen & Reeh, 1993; Lou *et al*., 1991; Jinno *et al*., 1994) *via* nicotinic cholinergic receptors on primary afferent nerve fibres (Flores *et al*., 1996). At very high concentrations (10^{-3}–10^{-2} M), nicotine induces plasma extravasation (PE) (Schilling *et al*., 1992), a component of the inflammatory response, and enhances histamine-induced PE (Myers *et al*., 1988). However, at lower concentrations (10^{-8}–10^{-5} M) nicotine administered alone has not been reported to cause any significant increase in PE and may even depress PE induced by either histamine (Mayhan & Sharpe, 1998) or bradykinin (BK) (Miao *et al*., 1992; 1997), both potent inflammatory mediators. Because opioids can depress PE (Green & Levine, 1992) we tested if opioid antagonists might unmask an action of nicotine to promote PE, in particular since effects of low-dose intrathecal nicotine, probably mediated by action on the central terminal of nociceptors (Roberts *et al*., 1995), can be potentiated by opioid antagonists (unpublished observations). We now report evidence indicating that nicotine can act at very low concentrations to promote PE, by an action at nociceptors, but that this effect is normally masked by opioids derived from the adrenal medulla.

Methods

Experiments were performed on male Sprague-Dawley rats (300–400 g, from Bantin and Kingman, Fremont, CA, U.S.A.). Knee joint perfusion was performed in pentobarbitone (50 mg kg^{-1}, i.p.) anaesthetized rats (Miao *et al*., 1992; 1997; Green & Levine, 1992). In brief, 10 min after injection of Evans blue dye (50 mg kg^{-1}, i.v.), which binds stoichiometrically to albumin (Plante *et al*., 1992), a 30-gauge needle was inserted into the knee joint for perfusion. After infusion of an initial volume of 100–200 μl vehicle (normal saline), an outflow needle (25-gauge) was inserted into the knee joint, approximately 3 mm from the inflow needle. Fluid was infused and

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withdrawn at a constant rate of 250 μl min^{-1} for 5-min intervals. Perfusate samples were analysed for the amount of Evans blue dye by spectrophotometric measurement of absorbance at 620 nm (Carr & Wilhelm, 1964). To assess effects of nicotine, PE was enhanced by infusion of BK, which results in a stable elevation in PE. This technique was employed since it results in a level of PE which permits detection of inhibitory as well as stimulatory actions on PE, both of which have been reported previously for nicotine (Myers *et al*., 1988; Mayhan & Sharpe, 1998; Miao *et al*., 1992; 1997).

Surgical and chemical ablation procedures

Bilateral transection of sciatic and saphenous nerves To determine whether any anti-inflammatory effect (i.e., decrease in PE) of intra-articular nicotine observed was mediated through a spinal reflex (Miao *et al*., 1992; 1997), in some experiments, the sciatic and saphenous nerves were bilaterally transected immediately before knee joint perfusion. After separating the biceps femoris and semitendinosus muscles at the posterior aspect of the thigh, the sciatic nerve was located by blunt dissection. It was cut at a level close to the sacral plexus (before it gives rise to the tibial and common peroneal nerves). The saphenous nerve was isolated from the adjacent vascular bundle on the medial aspect of the thigh and then cut at a level just superior to its trifurcation. Acute transection of sciatic and saphenous nerves did not affect the baseline level of BK-induced PE (data not shown).

Denervation of the adrenal medullae To study the contribution of the adrenal medullae to the effect of nicotine on BK-induced PE, the adrenal glands were denervated by removing the suprarenal ganglia (Miao *et al*., 1997; Araki *et al*., 1984; Celler & Schramm, 1981). Following lateral incisions in the abdominal wall, the suprarenal ganglia and the nerves innervating the adrenal glands were exposed. The nerves connecting to the suprarenal ganglia were cut and the ganglia removed. The removed suprarenal ganglia and the attached nerves to the adrenal medulla, to the major splanchnic nerve and to the minor splanchnic nerve were put on a slide and inspected after the operation; in this way the completeness of denervation of the adrenal medullae was controlled. Perfusion of the knee joints was carried out immediately after adrenal denervation (Miao *et al*., 1997). Bilateral adrenal nerve lesion did not affect the baseline level of BK-induced PE (data not shown).

Neonatal capsaicin treatment To study the involvement of primary sensory afferents, we treated a group of rats neonatally with the neurotoxin capsaicin, 100 mg kg^{-1}, s.c. on the second day after birth (Nagy *et al*., 1983); a loss of chemonociception was confirmed in the adult by the eye wipe test (Craft *et al*., 1993).

Statistics

Data are presented as per cent change from the plateau level of BK-induced PE, produced by nicotine (mean $\pm$ s.e.mean). Significant differences between time-effect curves of the control and of the treatment groups were determined by two-way repeated measures analysis of variance (ANOVA) followed by *post-hoc* Fisher's test. Differences between maximum responses produced by nicotine in the treatment and control group were determined by unpaired one-tailed Student's *t*-test. When comparing the treated knee with the contralateral control knee, differences were determined by paired one-tailed Student's *t*-test.

Materials

The following drugs were used: bradykinin acetate (160 ng ml^{-1}, intra-articularly), nicotine hydrogen tartrate, naloxone hydrochloride, naltrindole hydrochloride, nor-binaltorphimine hydrochloride, CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr), and hexamethonium chloride from Sigma Chemical Co., St. Louis, MO, U.S.A.. Phentolamine hydrochloride was from Ciba Pharmaceutical, Summit, NJ. RP-67,580 was a gift from Rhone Poulenc Rorer, France. Capsaicin was dissolved in EtOH and Tween 80 (1 : 1) and RP-67,580 was initially dissolved in EDTA (ethylenediaminetetraacetate; final concentration: $<0.2\%$). All other compounds were dissolved in normal saline.

Results

Effect of opioid receptor antagonist on the inhibitory action of intra-articular nicotine on BK-induced PE

Although having the potential to activate nociceptors and stimulate PE, intra-articular perfusion of nicotine (10^{-8} M) decreased BK-induced PE, compared to contralateral control knees perfused with BK alone (open circles vs crosses in Figure 1A, $F=5.0$, $P<0.05$). However, co-perfusion of naloxone (10^{-5} M) with nicotine (10^{-8} M) enhanced BK-induced PE (Figure 1A,$F=36.64$, $P<0.01$).

Effect of unilateral transection of sciatic and saphenous nerves on the action of opioid receptor antagonist

Immediately after unilateral de-afferentation, to isolate actions mediated by nociceptor terminals, intra-articular nicotine (10^{-8} M) administered with naloxone still enhanced BK-induced PE (filled squares in Figure 1B vs filled circles in Figure 1A,$F=5.00$, $P<0.05$).

Effect of selective antagonist for specific opioid receptor subtypes on the action of nicotine on BK-induced PE

We next assessed which opioid receptors are involved in the effect of naloxone. In knees in which naltrindole (δ-opioid receptor antagonist, 10^{-5} M) was co-perfused with nicotine (10^{-8} M), we observed enhancement of BK-induced PE, similar to that in the naloxone-treated knees (Figure 1C, $F=3.60$, $P>0.05$). Neither nor-binaltorphimine (κ-opioid receptor antagonist, 10^{-6} M) nor CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide; a μ-opioid receptor antagonist, 10^{-6} M) added to nicotine enhanced BK-induced PE (data not shown).

Effect of adrenal denervation on the action of opioid receptor antagonist

To determine the role of adrenal medulla-derived endogenous opioids in masking nicotine-induced increase in BK-induced

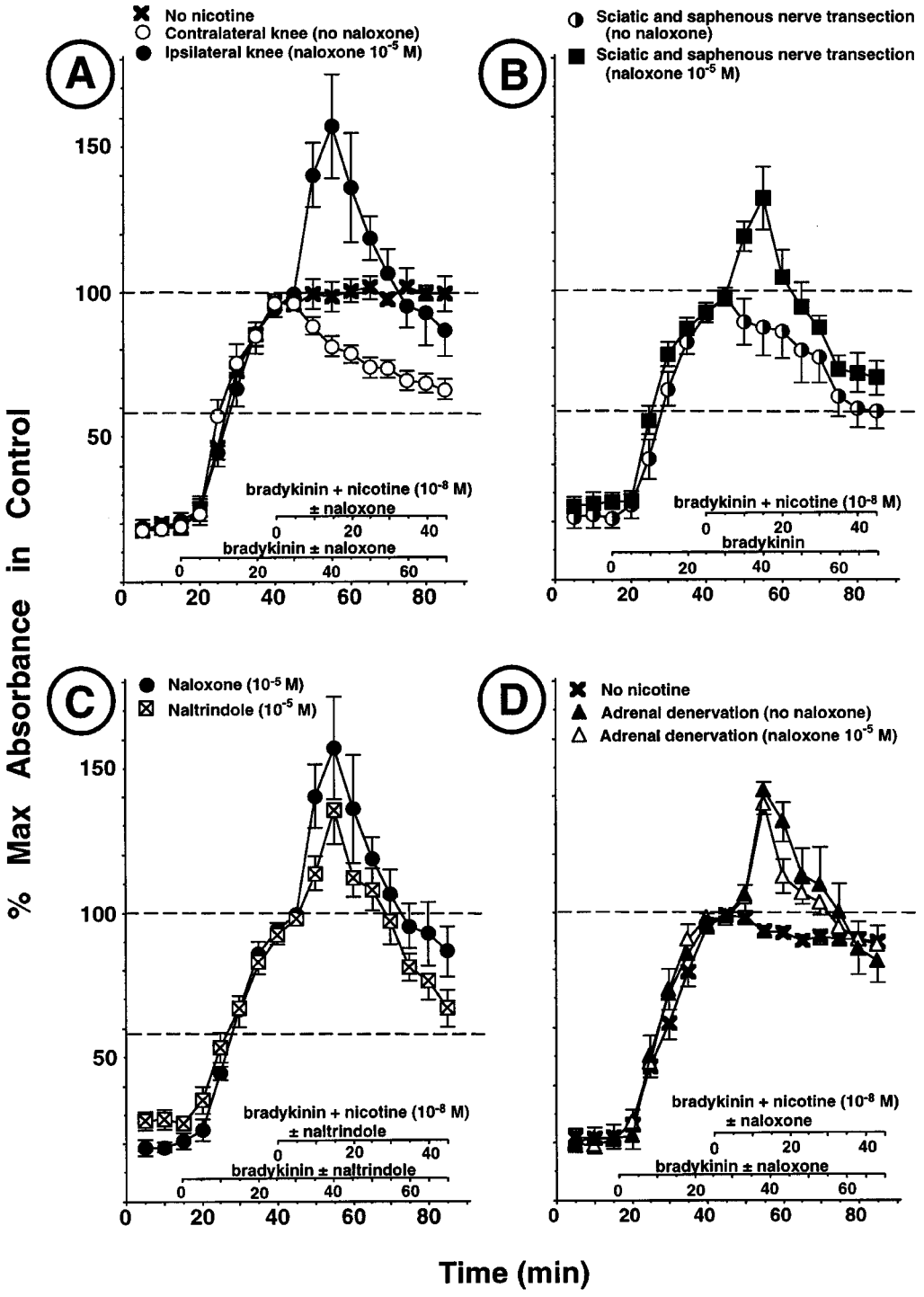

Figure 1 (A) Effect of intra-articular nicotine (10^{-8} M) and naloxone (10^{-5} M) on BK-induced PE. Co-perfusion of naloxone and nicotine enhanced BK-induced PE (●, $n=6$). In contralateral knees, nicotine alone decreased BK-induced PE (○, $n=6$). BK (160 ng ml^{-1}) alone showed stable PE over time (**X**, $n=8$). Naloxone alone had no effect (data not shown). In all figures, the upper dashed line represents the average level of PE induced by BK at plateau (i.e., **X**) while the lower one the maximal decrease in BK-induced PE after intra-articular nicotine at 10^{-8} M (i.e., half-filled circles in Figure 1B). *n*: number of experiments performed. (B) Effect of de-afferentation on nicotine (10^{-8} M)-induced inhibition of BK-induced PE and the enhancement of BK-induced PE by nicotine administered with naloxone (10^{-5} M). After acute bilateral transection of sciatic and saphenous nerves, naloxone plus nicotine could still enhance BK-induced PE (■, $n=6$). Half-filled circles represents nicotine-induced suppression of BK-induced PE in the absence of naloxone in nerve-intact animals ($n=10$). (C) Effect of naltrindole (a δ-opioid receptor antagonist) on intra-articular nicotine (10^{-8} M)-induced inhibition of BK-induced PE. In naltrindole (10^{-5} M, intra-articularly)-treated rats, nicotine enhanced BK-induced PE (⊠, $n=8$), similar to that in naloxone-treated rats (●, $n=8$). (D) Effect of adrenal denervation on intra-articular nicotine (10^{-8} M)-induced inhibition of BK-induced PE. Adrenal denervation did not affect the level of BK-induced PE (**X**, $n=8$). Compared to adrenal-intact rats receiving intra-articular nicotine (lower dashed line), the increase in BK-induced PE by nicotine in adrenal denervated rats is significantly enhanced (▲, $n=6$). Addition of intra-articular naloxone did not cause further enhancement (△, $n=6$).

PE, we evaluated the effect of denervation of the adrenal medulla on modulation of BK-induced PE by nicotine. When nicotine alone was perfused into the knee joint in adrenal denervated rats, it produced a very similar increase in BK-induced PE to that of nicotine plus naloxone in control animals (Figure 1D); naloxone exerted no further effect on this nicotine enhancement of BK-induced PE.

Effect of low-concentration nicotine on BK-induced PE in the presence of opioid receptor antagonist

We also employed lower concentrations of nicotine to determine the minimum concentration of nicotine having a proinflammatory action. Nicotine [10^{-10} M, but not 10^{-11} M (data not shown)] co-perfused with naloxone enhanced BK-induced PE (Figure 2A).

Effect of destruction of primary afferents on the action of nicotine

To determine whether the pro-inflammatory effect of nicotine was mediated by activation of nociceptors, we evaluated the effect of nicotine in rats treated neonatally with capsaicin. This treatment did not affect the nicotine-induced inhibition of BK-induced PE, but did abolish the nicotine-induced enhancement of BK-induced PE in the presence of naloxone (Figure 2B, $F=4.95$, $P<0.05$).

Effect of substance P receptor antagonist on the action of nicotine

To determine if neurokinin release from nociceptors is involved in the enhancing effect of nicotine on BK-induced PE we tested the effect of RP-67,580 (a NK-1 neurokinin receptor antagonist). Co-perfusion of RP-67,580 (10^{-7} M, intra-articularly), blocked the ability of nicotine (10^{-8} M) with naloxone to enhance BK-induced PE (Figure 2C, $F=31.29$, $P<0.01$). In control experiments, intra-articular nicotine and naloxone in the contralateral knee still enhanced BK-induced PE, similar to that without RP-67,580 (Figure 2C, $F=15.86$, $P>0.05$).

Effect of nicotinic receptor antagonist on the action of systemically-administered nicotine

To determine whether systemically administered nicotine, at doses consumed by smokers or nicotine patch users (10^{-5} mg kg^{-1}, s.c.), can increase BK-induced PE, nicotine was administered systemically. In knees perfused with naloxone, systemic nicotine (10^{-5} mg kg^{-1}, s.c.) enhanced BK-induced PE (filled triangles in Figure 2D). To determine if this systemic effect of nicotine was due to a local action in the knee, the contralateral knees were perfused with naloxone and hexamethonium (10^{-5} M). BK-induced PE was no longer enhanced after systemic administration of nicotine in knees treated with hexamethonium (Figure 2D, $F=31.49$, $P<0.01$).

Discussion

We demonstrate that at *sub*-nanomolar concentrations nicotine can enhance neurogenic inflammation by action at nociceptors. This enhancement is usually not observable, due to a tonic suppression by endogenous opioids. Specifically, unmasking of the enhancement of BK-induced PE produced by nicotine was observed in the presence of non-selective (or selective δ) opioid receptor antagonists. Adrenal denervation also uncovered this nicotine-induced action and occluded enhancement by naloxone, suggesting that the naloxone-sensitive inhibition of nicotinic receptors is mediated by endogenous opioids derived from the adrenal medulla. Supported by the reports that denervation of the adrenal medulla is known to result in a decrease in circulating opioids (Przewlocka *et al*., 1986; Mason *et al*., 1987; Chritton *et al*., 1991), our results provide evidence that endogenous release of opioids exert a tonic inhibitory action on the proinflammatory effect of low-dose nicotine. Because both opioid receptors (Taddese *et al*., 1995; Ma *et al*., 1997) and nicotinic receptors (Steen & Reeh, 1993; Boyd *et al*., 1991) are present on sensory neurons, it is likely that the actions we observed are mediated by interaction between receptors located on primary afferent nerve endings in the knee joint.

Physiological effects of nanomolar concentration of nicotine have previously been reported, including induction of dopamine release at 3×10^{-9} M (Rowell, 1995) and increase in expression of nerve growth factor (NGF) receptor at 10^{-8} M (Terry & Clarke, 1994). The present study now demonstrates a physiological effect of nicotine at a *sub*-nanomolar concentration [at a potency similar to that of ligands acting at other ligand-gated ion channels (Liu *et al*., 1996; Janaky *et al*., 1999)]. Since, it is likely that nicotinic receptors at the central terminals of primary afferents (Roberts *et al*., 1995) as well as those at central synapses may employ a similar mechanism to those in peripheral terminals, modulation by opioids of nicotinic activation may be of importance at many levels in the nervous system (Hamann & Martin, 1992). In fact, subdiaphragmatic vagotomy or intrathecal naloxone, both of which remove a tonic descending opioid-mediated inhibition in the spinal cord, results in a similar uncovering of a sensitivity to intrathecal administration of very low-dose nicotine (unpublished observations).

The subtype of nicotinic cholinergic receptor at which nicotine acts to stimulate sensory neurons remains to be determined. At least nine neuronal nicotinic cholinergic receptor subunits have been identified and cloned to date, each of different distribution and binding affinity for nicotinic cholinergic receptor ligands. The lowest K_d for nicotine, which is at the $\alpha_4\beta_2$ receptor, is 10^{-9} M (Briggs *et al*., 1997). Hargreave and colleagues have suggested that the nicotinic receptor on the primary afferent is $\alpha_3\beta_4$ (Flores *et al*., 1996); while $\alpha_3\beta_4$ has extremely high affinity (3×10^{-10} M) for epibatidine (a nicotinic agonist) (Parker *et al*., 1998), its K_d for nicotine has not been reported.

In summary, we have demonstrated an effect of sub-nanomolar nicotine on primary afferents, one that is tonically masked by adrenal medulla-derived opioids. The *in vivo* approach we have employed is an important tool for further investigating actions of nicotine, including analgesia that may occur at the central terminals of the primary afferent (Hamann & Martin, 1992; Caggiula *et al*., 1995). The present study also has important clinical implications because the pro-inflammatory effect of nicotine is exerted at concentrations detected even in people exposed only to second-hand

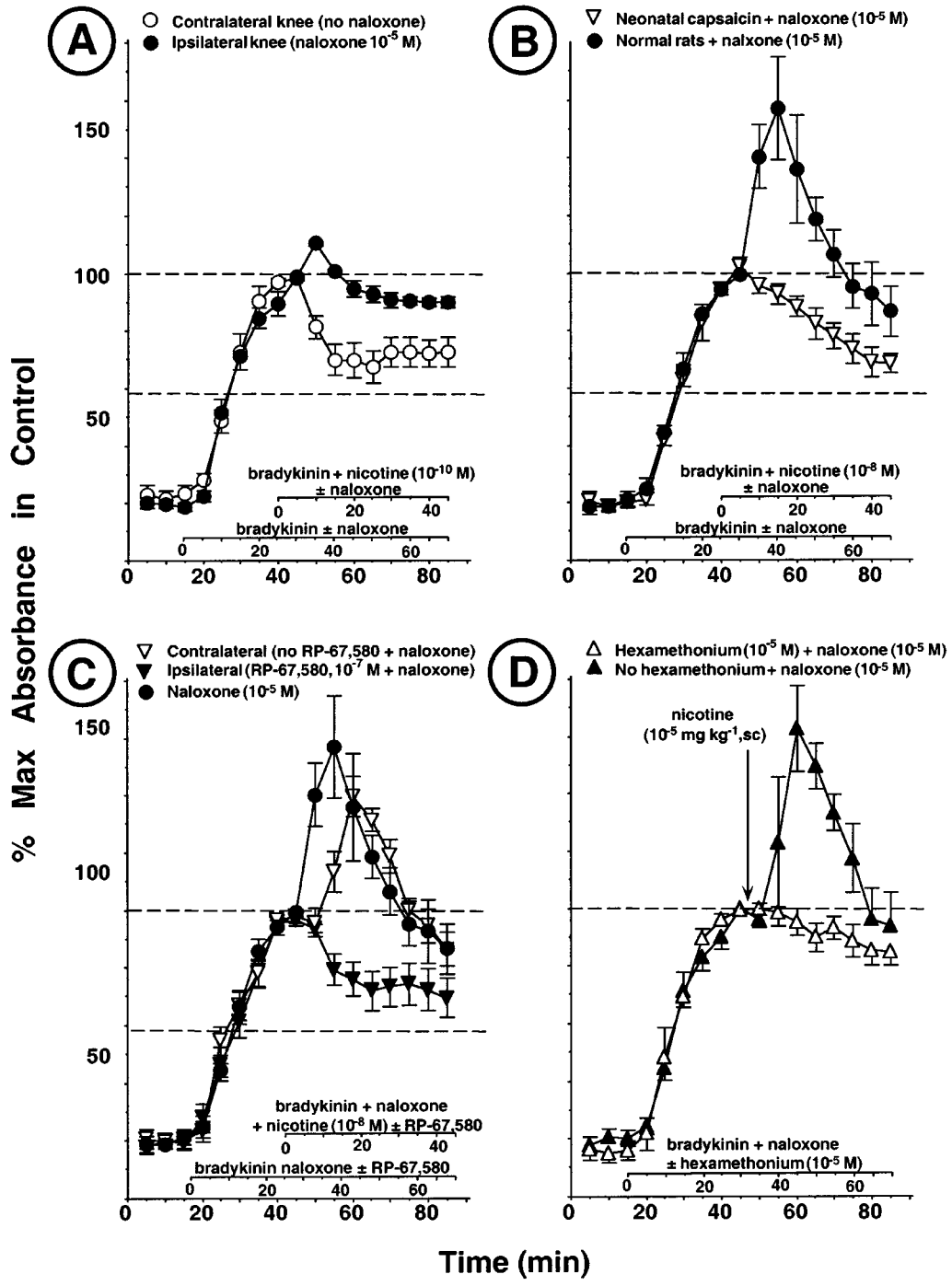

Figure 2 (A) Effect of naloxone on sub-nanomolar nicotine-induced inhibition of BK-induced PE. Co-perfused with naloxone (10^{-5} M), nicotine (10^{-10} M) enhanced BK-induced PE (●, $n=8$). In contralateral knees, nicotine generated a decrease in BK-induced PE (○, $n=8$). (B) In rats treated neonatally with capsaicin, intra-articular treatment with nicotine (10^{-8} M) and naloxone (10^{-5} M) is no longer able to enhance BK-induced PE (▽, $n=8$) compared to normal rats (●, $n=8$). (C) Effect of RP-67,580 (a neurokinin-1 receptor antagonist; 10^{-7} M, intra-articularly) on intra-articular nicotine (10^{-8} M)-induced inhibition of BK-induced PE. In the presence of naloxone (10^{-5} M), intra-articular RP-67,580 significantly attenuated the effect of nicotine (10^{-8} M) in enhancing BK-induced PE (▼, $n=8$) without affecting the response in the contralateral control knee (▽, $n=8$). In the presence of naloxone, nicotine enhanced BK-induced PE (●, $n=8$). (D) Effect of hexamethonium (a nicotinic receptor antagonist) on nicotine (10^{-5} mg kg^{-1}, s.c.)-induced inhibition of BK-induced PE. Intra-articular hexamethonium (10^{-5} M) abolished the enhancement of BK-induced PE produced by systemic nicotine and naloxone (10^{-5} M, intra-articularly) (△, $n=6$), without affecting the enhancement in the contralateral knees not perfused with hexamethonium (▲, $n=6$).

smoke (Bergman *et al*., 1996; Trout *et al*., 1998). Finally, it is not known, at this time, whether the findings of a proinflammatory effect at very low dose of nicotine should provide caution for the therapeutic use of nicotine for

analgesia (Decker & Meyer, 1999), and whether there are interactions between opioids used in the treatment of pain and nicotine.

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