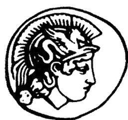

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Involvement of the endogenous nitric oxide signalling system in bradykinin receptor activation in rat submandibular salivary gland

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Abstract

Biochemical signalling events coupled to the bradykinin B_2-receptor subtype, related to nitric oxide and prostaglandin E_2 generation were studied in rat submandibular gland. Bradykinin stimulation of the B_2-receptor triggered activation of phosphoinositide turnover, translocation of protein kinase C, stimulation of nitric oxide synthase activity, increased production of cGMP and release of prostaglandin E_2. Bradykinin stimulation of nitric oxide synthase and cGMP production was blunted by agents able to interfere with calcium/calmodulin and phospholipase C activities, while a protein kinase C inhibitor was able to stimulate bradykinin action. Moreover, a specific B_2-bradykinin antagonist of the reversible nitric oxide synthase inhibitor abrogated the bradykinin stimulation of nitric oxide synthase activity, cGMP accumulation and prostaglandin E_2 generation. Furthermore, a specific inhibitor of phospholipase A_2 blocked the bradykinin-induced prostaglandin E_2 release. These results suggest that apart, from the direct effect of bradykinin as an inducer of vasopermeability, it also appears to be a vasoactive chemical mediator that triggers, through release of prostaglandin E_2, a feedback mechanism that induces a protective adaptation of the gland, modulating the course of inflammation. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Salivary gland; Bradykinin; Nitric oxide synthase; cGMP; Prostaglandin E_2; Phosphoinositide turnover; Protein kinase C

1. Introduction

Nitric oxide synthase immunoreactive neurones and also perivascular, periacinar and periductal nerve fibres have been demonstrated in submandibular salivary glands (Lohinai et al., 1995). In rat submandibular autonomic ganglia, virtually all neurones show nitric oxide synthase immunoreactivity (Ceccatelli et al., 1994).

Most ganglion cells contain vasoactive intestinal polypeptide and neuropeptide immunoreactivity, suggesting that autonomic neurones can produce not only the classical neurotransmitter but also several peptides and the gas nitric oxide (Modin, 1994). However, non-adrenergic, non-cholinergic vascular control via neuropeptides and nitric oxide has been described (Modin et al., 1994). The neuronal localization of nitric oxide synthase suggests that nitric oxide could

Abbreviations: L-NMMA, L-*N*-monomethyl arginine.

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control exocrine function and blood flow in the submandibular gland. In fact, the glands exhibit vasoregulation and nitric oxide-dependent basal vasodilator tone (Kerezoudis et al., 1993; Lohinai et al., 1996). The effect of nitric oxide on salivary amylase secretion has also been described (Lohinai et al., 1997).

Bradykinin, the main kinin cleaved from glandular kininogens, has a potent vasodilator effect. It increases secretory activity in some glands during inflammation via B_2-receptors (Bhoola et al., 1992). This receptor belongs to the family of peptide hormone receptors linked to G proteins (Bhoola et al., 1992). Moreover, prostaglandins and nitric oxide appear to mediate some of the effects of bradykinin. Kinins may also mediate part of the vasodilatation, oedema and pain observed during salivary gland inflammation, and can stimulate the release of cytokines from monocytes (Nasjletti and Malik, 1981).

It has proposed that bradykinin is released from endothelial cells during salivary gland inflammation as well as during myocardial ischaemia. The involvement of bradykinin in both inflammation and ischaemia appears to be related to the stimulation of nitric oxide release, which in turn increases the production of cGMP to provide protection (Vegh et al., 1991). However, there have been no attempts to determine the mechanism or possible pathway of signal transduction involved in bradykinin–B_2-receptor-dependent activation in submandibular glands.

Here we have examined the effects of the bradykinin activation of B_2-subtype receptors on the production of nitric oxide and cGMP in rat submandibular glands.

2. Material and methods

2.1. Nitric oxide synthase activity assay

Nitric oxide synthase activity was measured in submandibular glands of male Wistar rats using L-[U-^{14}C]arginine as a substrate (Bredt and Snyder, 1989). In brief, submandibular glands were carefully dissected and incubated in Krebs–Ringer bicarbonate solution containing 18.5 kBq of L-[U-^{14}C]arginine for 20 min before the addition of agonist. When inhibitors were used they were added from the beginning of the incubation time at the final concentrations indicated in the text. The incubations were carried out under a 50% CO_2 in oxygen atmosphere at 37°C and stopped by homogenization of the glands in 1 ml 20 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA and 1 mM dithiothreitol at 4°C. The supernatants were passed through 2 ml Dowex AG-50 WX-8 columns and L-[U-^{14}C]citrulline was eluted with water and quantified as described by Bacman et al. (1998). Nitric oxide synthase activity was inhibited by more than 90% by 0.5 mM L-NMMA. In the experiments with the activation of nitric oxide synthase by the agonist we chose lower concentrations of this arginine analogue to inhibit the effects, in order not to modify basal nitric oxide synthase activity.

2.2. cGMP assay

Glands were incubated in 1 ml Krebs–Ringer bicarbonate containing 0.1 mM isobutyl methyl xanthine for 30 min under constant current of 5% CO_2 in oxygen. The agonist was added in the last 10 min while inhibitors were included in the incubation volume from the beginning. Reactions were stopped by homogenization as previously stated (Bacman et al., 1998) and samples were radioimmunoassayed using [^{125}I]cGMP from Dupont New England Nuclear (81,400 kBq/mmol) and anti-cGMP antiserum from Sigma Chemical Co. (St Louis, MO, USA).

2.3. Protein kinase C activity

Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from [^{32}P]-ATP into histone H_1, as described by Borda et al. (1998). In brief, incubations were conducted for 30 min at 30°C in a final volume of 85 μl. In final concentrations, the assay mixture contained 25 μmol ATP (14.8 kBq), 10 mM magnesium acetate, 5 mM β-mercaptoethanol, 50 μg histone H_1, 20 mM HEPES, pH 7.4, and, unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 μg/ml of phosphatidylserine vesicles. The incorporation of [^{32}P]phosphate into histone H_1 was linear for at least 30 min. The reaction was stopped by the addition of 2 ml ice-cold 5% trichloroacetic acid, 10 mM H_3PO_4. The radioactivity retained on GF/c glass-fibre filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. Protein kinase C activity was determined after subtracting the incorporation in the absence of calcium and phospholipids. The data were expressed as pmol of phosphate incorporated into the substrate per minute per milligram of protein (pmol/min per mg protein).

2.4. Measurement of total labelled inositol phosphates

Submandibular glands were incubated for 120 min in 0.5 ml of Krebs–Ringer bicarbonate gassed with CO_2 in oxygen with 37 kBq [myo-^{3}H]inositol (sp. act. 533 kBq/mmol) from Dupont New England Nuclear; LiCl (10 mM) was added to determine the accumulation of inositol monophosphate according to the technique of Berridge et al. (1982). Agonist was added 30 min before the end of the incubation period and the blockers 30 min before the addition of agonist. Water-

soluble inositol phosphates were extracted after 120 min incubation following the method of Berridge et al. (1982). Submandibular gland slices were quickly washed with Krebs–Ringer bicarbonate and homogenized in 0.3 ml of Krebs–Ringer bicarbonate with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 ***g*** for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7-ml column of Bio-Rad AG (formate form) 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid, which had been previously washed with 10 mM Tris–formic, pH 7.4. The resin was then washed with 20 vol of 5 mM myo-inositol followed by 6 vol of water, and inositol phosphates were eluted with 1 M ammonium formate in 0.1 M formic acid. Fractions (1 ml) were recovered and their radioactivity determined by scintillation counting. Peak areas were determined by triangulation. Results corresponding to the second peak were expressed as absolute values of area units under the curve per milligram of wet weight tissue (area/mg tissue wet wt) following the criteria of Simpson's equation (Borda et al., 1998). In order to confirm the absence of $[^3H]$myoinositol in the eluted peaks of inositol phosphates, chromatography was performed on silica-gel 60 F254 sheets (Merk), using propan-2-ol/6N NH_4OH (14:5) as the developing solvent following the procedure of Hokin-Neaverson and Sadeghian (1976).

2.5. Prostaglandin E_2 assay

Submandibular glands were incubated for 30 min in 1 ml of Krebs–Ringer bicarbonate, gassed with 5% CO_2 in oxygen at 37°C. Agonist was added in the last 3 min and, when inhibition experiments were done, the inhibitors were included in the incubation medium from the beginning. Supernatants were obtained by centrifugation for 15 min at 800 ***g***; ethyl acetate extracts were dried under a nitrogen stream and the residue was resuspended in the appropriate assay buffer. Prostaglandin E_2 was determined by a radioimmunoassay kit for $[^{125}I]$-PGE_2 (Dupont New England Nuclear). Results were expressed in picograms per milligram tissue wet weight (pg/mg).

2.6. Drugs

Bradykinin, L-NMMA, L-arginine, verapamil, staurosporine, trifluoperazine and sodium nitroprusside (Sigma Chemical Co., St Louis, MO, USA); HOE 140 (RBI, Natick, MA, USA), 4-(4-octadecyl)-4-oxobenzenebutenoic acid (benzenebutanoic acid) and U-73122 (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA). Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted to achieve the final concentrations stated in the text.

2.7. Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, the Student–Newman–Keuls test was applied after ANOVA. Differences between means were considered significant if $p < 0.05$.

3. Results

In order to evaluate the effect of bradykinin on submandibular salivary glands, intracellular signals such as nitric oxide synthase, inositol phosphate accumulation, protein kinase C traslocation, endogenous cGMP production and prostaglandin E_2 generation were measured. For this purpose, submandibular glands exposed to bradykinin were assayed at different times to determine accurately their kinetic behaviour and the time for their maximal effect.

As shown in Fig. 1(A), there was a dose-dependent increase in nitric oxide synthase activity when tissues were exposed to different concentrations of bradykinin. The maximal increment of nitric oxide synthase activity induced by each concentration of the neuropeptide was obtained at 15 min. L-NMMA (1×10^{-5} M), a reversible nitric oxide synthase inhibitor, blunted the stimulatory action of bradykinin, while the natural substrate L-arginine (5×10^{-5} M) completely reversed the inhibition [Fig. 1(A)]. Additionally, the action of bradykinin could be blocked by the bradykinin antagonist HOE 140 (5×10^{-6} M), indicating the participation of submandibular B_2-subtype receptors [Fig. 1(B)]. The activation of nitric oxide synthase was accompanied by an increase in cGMP production (Table 1).

To determine the mechanism by which bradykinin stimulates nitric oxide synthase–cGMP production, tissue was incubated with different inhibitors of enzymatic pathways involved in B_2-bradykinin-receptor activation. Table 1 shows that the inhibition of phospholipase C by U-73122 (5×10^{-6} M) blocked the stimulatory action of bradykinin on both nitric oxide synthase activity and cGMP production. Moreover, the inhibition of calcium/calmodulin by trifluoparazine (5×10^{-6} M) and the inhibition of calcium influx by verapamil (1×10^{-6} M) significantly abrogated the action of bradykinin upon nitric oxide synthase and cGMP. In contrast, the inhibition of protein kinase C by staurosporine (1×10^{-9} M) potentiated the stimulatory effect of the neuropeptide (Table 1). In addition, the inhibition of phospholipase A_2 and cyclo-oxygenase also interfered with bradykinin-increased nitric

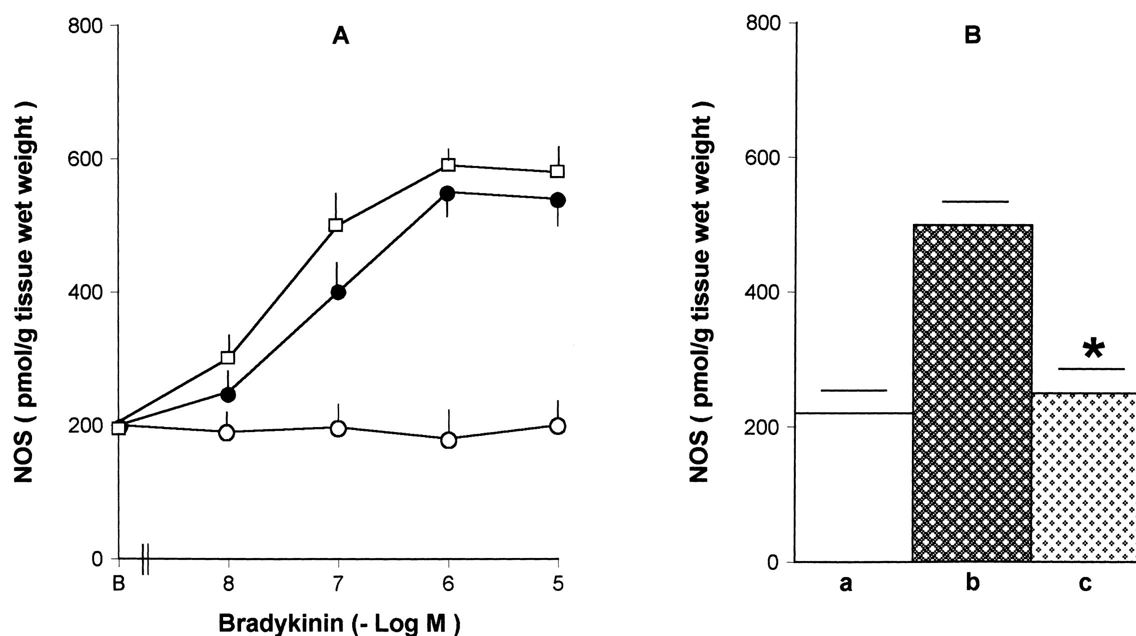

Fig. 1. Bradykinin (BK) action on nitric oxide synthase (NOS) activity. (A) Rat submandibular gland incubated with different concentrations of BK in the absence (●) or presence (○) of L-NMMA (1×10^{-5} M); reversal of inhibition by L-arginine (5×10^{-5} M) (□). (B) Rat submandibular glands incubated with 1×10^{-6} M BK alone (b) or in the presence of 5×10^{-6} M HOE 140 (c); basal values before the addition of any drugs (a). Values are mean ± SEM of eight experiments performed in duplicate. *Differ significantly from (b) ($p < 0.001$).

oxide synthase activity and cGMP accumulation (Table 1).

Furthermore, bradykinin was able to increase the accumulation of inositol phosphates (Fig. 2); this effect was inhibited by HOE 140 (5×10^{-6} M) and by U-73122 (1×10^{-6} M) [Fig. 2(A), (B)]. These results point to the participation of B_2-receptors and phospholipase C activation in the bradykinin-increased formation of inositol phosphates.

As can be seen in Fig. 3, bradykinin was also able to translocate protein kinase C from the cytosol to the membrane, an effect that was also abrogated by the specific B_2-antagonist.

To assess if an endogenous nitric oxide signalling

Table 1
Effect of bradykinin (BK) on nitric oxide synthase (NOS) activity and cGMP accumulation: influence of different inhibitory agents[a]

Additions	NOS (pmol/g tissue wet wt)	cGMP (pmol/g tissue wet wt)
Basal	265 ± 16	23 ± 2
BK	583 ± 22*[b]	63 ± 5*
BK + U-73122	258 ± 15	29 ± 3
BK + trifluoperazine	279 ± 16	32 ± 5
BK + verapamil	234 ± 12	29 ± 4
BK + staurosporine	879 ± 31**	98 ± 5**
BK + benzenebutenoic acid	252 ± 15	36 ± 3
BK + indomethacine	270 ± 17	37 ± 4

[a] NOS activity and cGMP accumulation were measured in rat submandibular glands incubated with or without inhibitors for 20 min and then for an additional 10 min with 1×10^{-6} M BK. Results are mean ± SEM of five experiments performed in duplicate in each group. Final concentrations of inhibitors were: U-73122 5×10^{-6} M, trifluoperazine 5×10^{-6} M, verapamil 1×10^{-5} M, benzenebutenoic acid 5×10^{-6} M and staurosporine 1×10^{-9} M.

[b] *Differ significantly from basal values ($p < 0.001$); **differ significantly from BK action ($p < 0.001$).

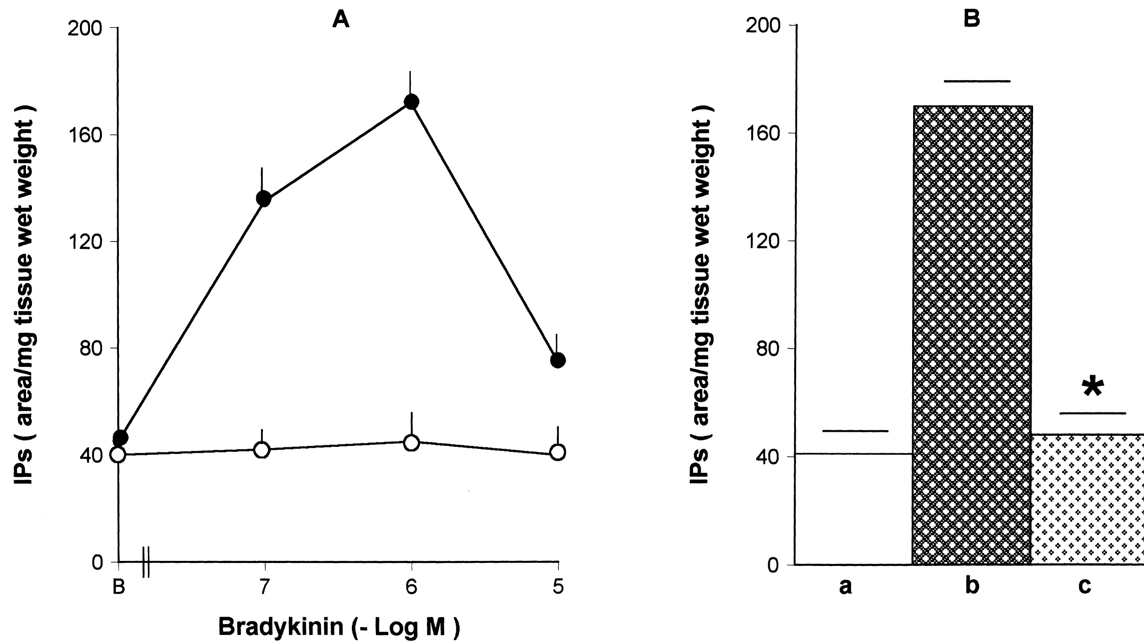

Fig. 2. Bradykinin (BK) action on the accumulation of inositolphosphates (IPs). (A) Increased IPs turnover by different concentrations of BK alone (●) or in the presence of 5×10^{-6} M HOE 140 (○). (B) Inhibition of the effect of 1×10^{-6} M BK (b) by treatment with 5×10^{-6} M U-73122 (c); basal values (a). Values are mean ± SEM of six experiments performed in duplicate in each group. *Differ significantly from (b) ($p < 0.001$).

system participates in the activation of phospholipase A_2 by bradykinin, the generation of prostaglandin E_2 by submandibular glands in the presence of bradykinin was measured. As can be seen in Fig. 4(A), the neuropeptide increased the release of prostaglandin E_2 in a concentration-dependent manner, the effect being blunted by HOE 140 and by L-NMMA. In addition, the generation of nitric oxide by sodium nitroprusside was able to mimic the effect of bradykinin on prostaglandin release. As a control, a phospholipase A_2 inhibitor, benzenebutenoic acid (5×10^{-6} M), abrogated the generation of the prostaglandin via the action of bradykinin [Fig. 4(B)].

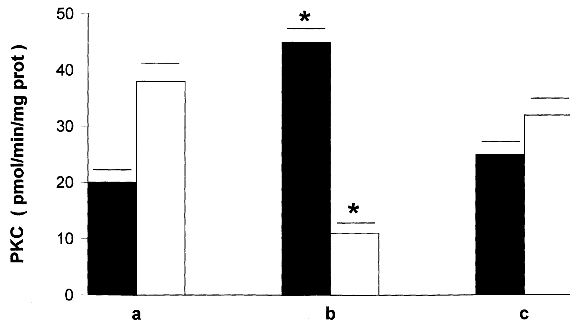

Fig. 3. Activation of rat submandibular gland protein kinase C (PKC) by bradykinin (BK). PKC activity was determined in both membrane (■) and cytosol (□). The effect of 1×10^{-6} M BK alone (b) or in the presence of 5×10^{-6} M HOE 140 (c) above basal values (a) was analysed. Results are mean ± SEM of six experiments performed in duplicate in each group. *Differ significantly from (a) ($p < 0.001$).

4. Discussion

These data provide important new insights into the pathways by which bradykinin induces rapid activation of nitric oxide synthase with subsequent accumulation of cGMP and the release of prostaglandin E_2 in rat submandibular gland. These actions of bradykinin appear to be specific, as they were abolished by the stereospecific inhibitor of nitric oxide synthase, L-NMMA (Mulsh and Busse, 1990), and could be reversed by the addition of the enzyme substrate L-arginine. Moreover, they seemed to be mediated by the activation of B_2-receptors, as HOE 140 (Goto et al., 1995) abrogated the bradykinin effects. In support of this, it has been suggested that bradykinin acting at B_2-receptors regulates the constitutive nitric oxide synthase (Lundberg, 1996). The mechanism of B_2-receptor stimulation by bradykinin in salivary glands appears to involve an increase in the hydrolysis of

phosphoinositide; intermediates from this could activate calcium/calmodulin-dependent constitutive nitric oxide synthase, as various agents known to interfere with calcium mobilization inhibit the activation of nitric oxide synthase activity by bradykinin. Elevation of intracellular calcium concentration increases the activity of constitutive nitric oxide synthase in many tissues (Sterin-Borda et al., 1995). However, intermediates of phosphoinositide hydrolysis (inositol triphosphate and diacylglycerol) oppose bradykinin-induced nitric oxide production. Thus, rapid activation of nitric oxide synthase is related to calcium mobilization; while inhibition of nitric oxide synthase requires extensive translocation of protein kinase C. This statement is based on the observed potentiation by inhibitors of protein kinase C of the effect of bradykinin on glandular nitric oxide synthase activity. Moreover, bradykinin causes translocation of protein kinase C from the cytosol to the cell membrane in submandibular glands, as it does in neuroblastoma NCB-20 cells (Fu et al., 1989). These findings may indicate cross-talk between the two enzymatic pathways: bradykinin B_2-receptor-coupled intracellular signals in rat submandibular gland appear to be increased, where calcium/calmodulin acts as an enzyme cofactor leading to the activation of nitric oxide synthase and protein kinase C downregulates its activity.

Among the mechanisms involved in the B_2-receptor-dependent activation of nitric oxide is the stimulation of soluble guanylate cyclase to increase the accumulation of cGMP. This is similar to the effect of others agonists (Sterin-Borda et al., 1995), suggesting that some of the physiological effect of nitric oxide may be mediated by the activation of guanylate cyclase.

Nitric oxide and cGMP have been suggested to act in salivary vasoregulation by several groups of investigators. The vessels of submandibular (Edwards and Garret, 1993; Kerezoudis et al., 1993) and parotid (Lohinai et al., 1996) glands exhibit an nitric oxide-dependent basal vasodilator tone. Moreover, the vasculature of the submandibular gland is highly responsive to exogenous nitric oxide. Therefore, the release of endogenous nitric oxide by bradykinin during inflammation may also affect the vascular conductance.

Another possible pathway involved in the action of bradykinin on the submandibular gland is by activation of cyclo-oxygenase. We have shown that the neuropeptide was able to release prostaglandin E_2 in a concentration-dependent manner, and this effect was inhibited by a specific B_2-antagonist. The fact that

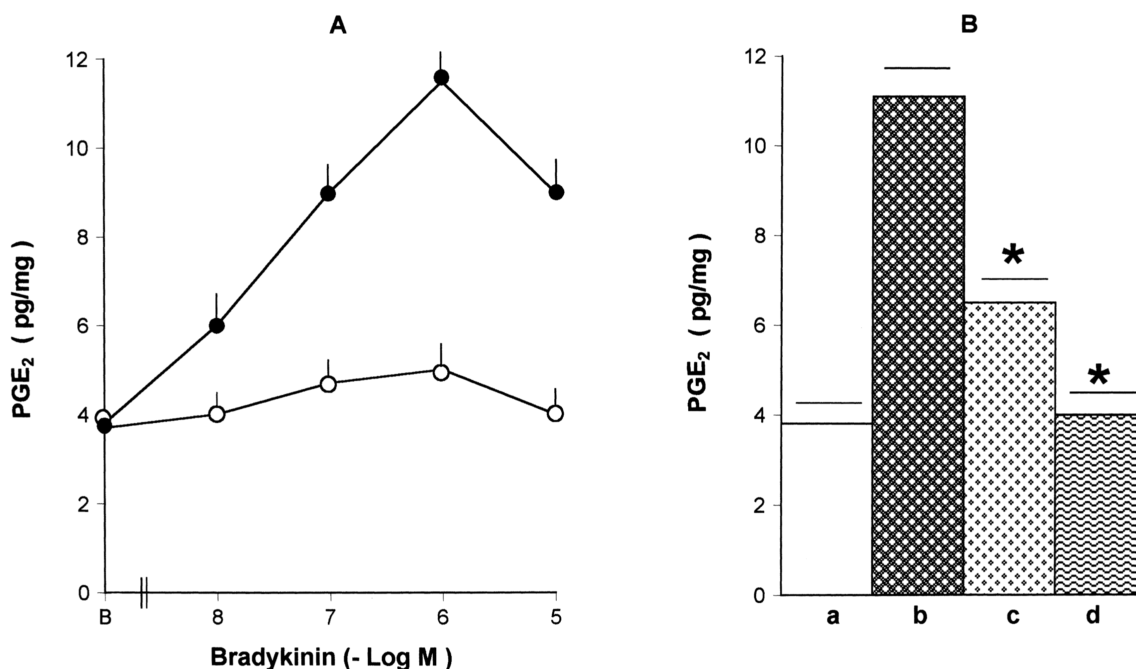

Fig. 4. Bradykinin (BK) action on prostaglandin E_2 (PGE_2) generation. (A) Increased release of PGE_2 by different concentrations of BK (●); effect of 1×10^{-5} M L-NMMA (○) or 5×10^{-6} M HOE 140 (▲). (B) Inhibition of the action of 1×10^{-6} M BK (b) by 5×10^{-6} M 4-(4-octadecyl)-4-oxobenzenebutenoic acid (c); the stimulatory action of 1×10^{-5} M sodium nitroprusside (d) upon basal values (a) is also shown. Values are mean ± SEM of five experiments performed in duplicate. *Differ significantly from (b) ($p < 0.001$).

blockers of phospholipase A_2 and nitric oxide synthase inhibit the release of prostaglandin E_2 by bradykinin strongly suggests that the nitric oxide-mediated pathway is involved in the increased metabolism of arachidonic acid and formation of prostaglandin E_2 resulting from bradykinin activity.

Prostaglandin E_2 is a local mediator of inflammation and it can also suppress diverse effector systems of inflammation (Zurier, 1990). Thus, apart from the direct action of bradykinin as the most important inducer of vasopermeability, it appears to be a vasoactive chemical mediator, initiating inflammatory reactions. However, the generation of prostaglandin E_2 by bradykinin in rat submandibular gland triggers a feedback mechanism reducing the stimulus modulating the course of inflammation, inducing a protective adaptation.

Further experiments are needed to evaluate the implication of all these mechanisms in salivary gland inflammation.

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