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Atrial natriuretic factor intracellular signaling in the rat submandibular gland

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ABSTRACT

We previously reported that intravenously administered atrial natriuretic factor (ANF) induced no salivation but enhanced agonist-evoked secretion in submandibular glands. The gene expression of ANF and natriuretic peptide receptors (NPR) was later reported in the glands. In the present study we sought to establish the intracellular signalling mechanisms underlying ANF modulation of salivary secretion. Fasted rats were prepared with submandibular duct and femoral cannulation. Dose–response curves to methacholine (MC) and norepinephrine (NE) were performed in the presence of cANP (4–23 amide) (selective NPR-C agonist) and ANF. Local injection of the agonist or ANF-induced no salivation, but enhanced MC and NE-evoked secretion. ANF and cANP (4–23 amide) enhanced phosphoinositide turnover being the effect abolished by U73122 (PLC inhibitor). Further ANF and cANP (4–23 amide) decreased basal cAMP content but failed to affect isoproterenol or forskolin-evoked cAMP. ANF response was inhibited by pertussis toxin and mimicked by cANP (4–23 amide) strongly supporting NPR-C activation. ANF-induced cAMP reduction was abolished by PLC and PKC inhibitors. The content of cGMP was dose dependently stimulated by ANF but not modified by cANP (4–23 amide). These findings support that ANF through NPR-C receptors coupled to PLC activation and adenylyl cyclase inhibition interacts with sialogogic agonists in the submandibular gland to potentiate salivation.

1. Introduction

The submandibular gland is one of the major salivary glands together with the parotid and the sublingual glands. In addition, hundreds of minor salivary glands are scattered over most of the oral cavity. Salivary glands are composed of two epithelial cell types, the acinar cells that secrete the salivary fluid as well as most of the salivary proteins and the ductal cells that secrete some proteins and modify the composition of primary saliva. The transepithelial movement of chloride is the major driving force for fluid and electrolyte secretion by salivary gland acinar cells [1]. Salivation is under the synergic control of both branches of the autonomic nervous system. The parasympathetic nervous system stimulates the secretion through impulses in the chorda tympani nerve that innervates it by releasing acetylcholine that evokes salivary secretion through the activation of muscarinic receptors mainly of the M3 subtype [2]. The sympathetic nervous system controls salivary secretion by α- and β-adrenergic receptor activation that also induces salivation. β-adrenergic stimulation evokes a secretion with a higher content of proteins. Several peptides and neuropeptides also modulate saliva formation affecting its final composition. Moreover some of them are locally produced within the glands and act as paracrine and/or autacrine factors, although others are released to saliva or blood stream [3].

Atrial natriuretic factor (ANF) is mainly released by mammalian atrial cardiocytes in response to a mechanical (atrial stretch) or neuroendocrine stimuli (α-adrenergic stimulation or endothelin-1) and plays a relevant role in the regulation of the cardiovascular and renal functions [4]. Although the heart is the major source of ANF, extra cardiac sites of production have been reported like the central nervous system, gastrointestinal tract, stomach, and the salivary glands [5–9]. However, the mechanisms that induce the release from these sources remain poorly understood, but it has been reported that in the stomach ANF is released by cholinergic and PACAP neural pathways [10]. ANF gene expression in the gastrointestinal tract varies according to fasting and feeding status strongly supporting that this peptide is involved in gastrointestinal physiology [8]. Several studies show that ANF likely acting as a paracrine factor influences digestive secretions and gastrointestinal motility. ANF modulates bile, pancreatic, and intestinal and salivary secretion in the rat [11–18]. The finding that ANF and natriuretic peptide receptors are expressed in the salivary glands suggests that ANF may act as a paracrine or autocrine factor to modulate the gland function [9]. We previously reported that ANF is not a sialogogic agonist when intravenously administered, but

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it enhances the secretory response of both parotid and submandibular glands when stimulated by muscarinic (methacholine), α-adrenergic (methoxamine) and peptidergic (substance P) agonists [15]. Further ANF also modifies the electrolyte excretion pattern induced by these agonists in both glands [16]. In the submandibular gland when salivation is evoked by agonists signalling through the phosphoinositide (PI) pathway ANF decreases sodium and increases potassium excretion suggesting that the atrial peptide acts also at the ductal level [16]. Although in the parotid gland ANF fails to modify fluid secretion induced by isoproterenol (β-adrenergic agonist) it potentiates isoproterenol-evoked amylase output through the activation of the PI pathway [17].

Natriuretic peptide receptors (NPR-A, NPR-B and NPR-C) are widely distributed and display distinct affinities for the members of the natriuretic peptide family [19]. ANF preferentially binds to NPR-A and NPR-C. Both NPR-A and NPR-B are membrane guanylyl cyclases (GC) with a tyrosine kinase domain [20]. Stimulation of NPR-A and NPR-B causes a rapid increase in cGMP which in turn activates downstream effectors such as cGMP dependent protein kinases, cGMP-gated ion channels and cGMP sensitive phosphodiesterases in order to bring about the physiological responses. The GC receptors mediate most of the renal and cardiovascular effects of the natriuretic peptides [4,20]. The NPR-C was originally considered a biologically silent receptor involved in the clearance of bound ligands by internalization and degradation [21]. However later studies showed that the NPR-C is a G-protein coupled receptor with an intracellular domain of 37 amino acids and devoid of GC and kinase activities. The domain is coupled through an inhibitory guanine nucleotide regulatory protein (Gi) to adenylyl cyclase (AC) inhibition and/or to PLC activation [21–24]. Activation of NPR-C results in decreased cAMP formation as well as increased DAG and IP3 generation from phosphatidyl inositol biphosphate which are involved in PKC activation and intracellular calcium mobilization, respectively [21]. This receptor subtype that signals through Gi is the predominant natriuretic peptide receptor in visceral and vascular smooth muscle cells [24,25]. In addition, in the submandibular gland NPR-C expression is very high; being only two fold less than in the lungs, an organ known for its abundant expression of this receptor subtype [9].

As the intravenous administration of ANF enhances the salivary secretion evoked by sialogogic agonists, in the present study we sought to establish the intracellular signalling pathways underlying this interaction.

Results showed that in the submandibular gland the local administration of cANP (4–23 amide) (selective NPR-C agonist) evoked no salivation but stimulated evoked salivary secretion through NPR-C receptors coupled to the activation of PLC and the inhibition of adenylyl cyclase supporting local modulation of cholinergic and adrenergic salivation by ANF.

2. Materials and methods

Sprague Dawley strain rats (School of Pharmacy and Biochemistry, University of Buenos Aires) weighing between 250 and 300 g were used in the experiments. The rats were housed in steel cages and maintained at 22–24 °C in a controlled room with 12-h light/dark cycle (light from 7:00 to 19:00 h) with free access to food and tap water. Rats were fasted for 14 h before experimental procedures to avoid variations in salivary secretion. All experiments were conducted following the recommendations of the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Publication 1985, revised 1996).

The following drugs were used: ANF and cANP (4–23 amide) (American Peptide, USA); KT-5023, U-73122 and GF-109203X (Calbiochem, CA, USA); methacholine, norepinephrine, pertussis toxin, isoproterenol, forskolin, chloralose and 3-isobutyl-1methyl-xanthine (IBMX) (Sigma MO, USA); [^{3}H] cAMP and Myo-[^{3}H] inositol (Amersham Biosciences, England). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

2.1. Salivary secretion experiments

In the present study we aimed to determine whether the NPR-C receptor mediated ANF response in the submandibular gland by using the selective agonist cANP (4–23 amide). Rats were prepared as previously described [15,16]. Briefly, animals were anesthetized with chloralose (100 mg/kg.0.5 ml NaCl (0.9%)). The right femoral vein was cannulated with a polyethylene catheter (P40 catheter, Rivero & Cia, Argentina) to administer the sialogogic agonists used in the study. Through a midline incision in the neck the trachea was intubated and the ducts of submandibular glands were exposed and cannulated with a fine glass cannula to collect saliva samples. No basal salivation was observed from the glands. Salivary secretion was induced by the administration of methacholine (MC) (1, 3 and 10 μg/kg) and norepinephrine (NE) (3, 10 and 30 μg/kg). Dose response curves to the sialogogic agonists were performed in the presence or in the absence of cANP (4–23 amide) administered into the gland and saliva samples were collected for 3 min. The secretory response to the agonists subsided in less than 3 min after the injection and 3 additional min were allowed until the administration of the next dose. Following the dose response curve and after a resting period of 30 min, cANP (4–23 amide) or ANF were administered into the

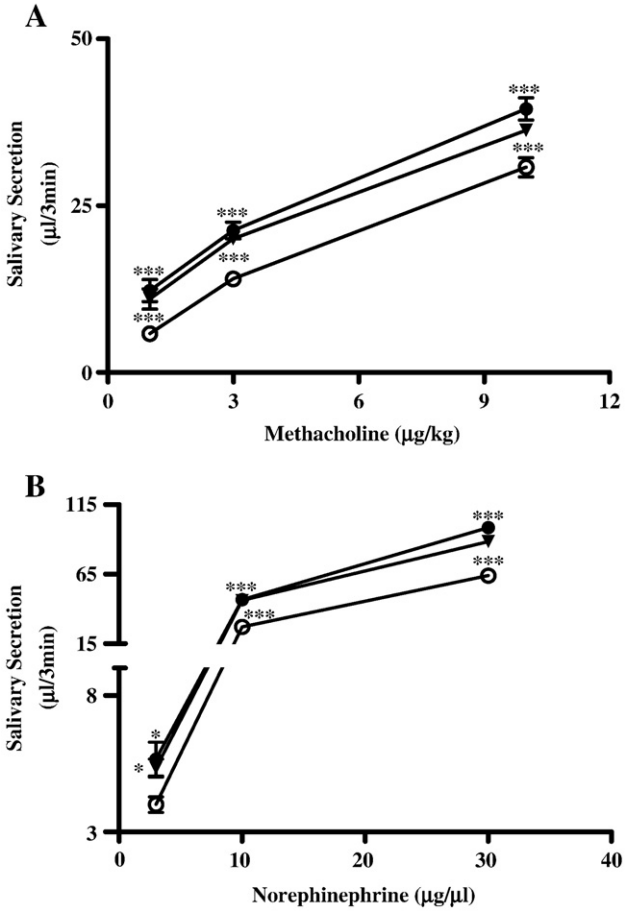

Fig. 1. A) Effect of cANP (4–23 amide) and ANF on cholinergic-evoked salivation in the submandibular gland. Salivary secretion was stimulated by methacholine (○) (1, 3 and 10 μg/kg) in the presence of cANP (4–23 amide) (●) (selective NPR-C receptor agonist) or ANF (▼) ***: $p<0.001$ vs. methacholine. Number of experiments: 6–8. B) Effect of cANP (4–23 amide) and ANF on adrenergic-evoked salivation in the submandibular gland. Salivary secretion was stimulated by norepinephrine (○) (3, 10 and 30 μg/kg) in the presence of cANP (4–23 amide) (●) (selective NPR-C receptor agonist) or ANF (▼). *: $p<0.05$ and **: $p<0.01$ vs. norepinephrine. Number of experiments: 6–8.

submandibular gland and then another dose response curve to the same agonist was performed in the same animal. Salivary samples were collected for 3 min in pre-weighed vessels and the quantity of secretion was determined by weighing. Results were expressed as µl/3 min, assuming saliva density as 1.0 g/ml. In the present study ANF was administered through the same route as cANP (4–23 amide) for comparative purposes with present and previous findings.

2.2. cAMP and cGMP determination

Studies were performed in submandibular gland slices. Animals were killed by cervical dislocation and the submandibular glands removed and detached from free connective and fat tissue. One millimeter thick sliced submandibular gland were pre-incubated for 15 min in a modified Krebs–Henseleit bicarbonate buffer of the following composition: 118 mM NaCl, 4.7 mM KCl, 1 mM $NaHPO_4$, 1.1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.5 mg/ml D-glucose supplemented with minimum essential amino acid solution and Basal medium Eagle vitamin solution (Biomedicals, CA, USA). The medium was equilibrated with 95%O_2–5%CO_2 and adjusted to pH 7.40. The accumulation of cAMP was assessed as previously described [12,13,26]. Briefly, tissues were incubated for 3 min in Krebs solution containing 1 mM IBMX and then for 12 min with ANF (1 pM as well as 0.1, 1, 10 and 100 nM), 100 nM isoproterenol or 100 nM cANP (4–23 amide). In another set of experiments tissues were exposed to 20 µM forskolin (AC activator) or pre-treated for 10 min with 10 µM U73122, 100 nM GF109203X or 2 µM KT-5023 (PLC, PKC and PKG inhibitors) before the addition of ANF or cANP (4–23 amide). In all experiments the reaction was stopped by homogenization in ice-cold absolute ethanol and centrifuged for 15 min at 1200 g. The supernatant was dried and the residue suspended for cAMP determination that was assessed by competition of [^{3}H] cAMP for PKA [26]. Results were expressed as pmol/mg protein. Proteins were measured as described by Lowry et al. [27]. In other experiments submandibular slices were incubated in a KHM in the presence or in the absence of *pertussis toxin* for 2 h at 37 °C and gassed with 95%O_2–5%CO_2 [13]. Tissues were then washed twice in the suspension medium and incubated with 100 nM ANF or 100 nM cANP (4–23 amide) to determine cAMP accumulation as detailed above. Results were expressed as percentage of the control value as in previous studies [13].

The content of cGMP was determined in the presence of ANF (1 pM–100 nM) and 100 nM cANP (4–23 amide) by radioimmunoassay as previously detailed [28]. Accumulation of cGMP was expressed as pmol/mg protein.

2.3. PI hydrolysis measurement

PI turnover was determined as previously described [12,17,29]. Briefly, submandibular gland slices were incubated in KHB containing 4 µCi/ml myo-[3H] inositol for 120 min. Tissues were washed and further incubated with 10 nM LiCl followed by the incubation with ANF (1 pM–100 nM) or cANP (4–23 amide) (100 nM) for 30 min. Tissues were then washed twice with fresh cold KHB solution and homogenized with chloroform-methanol (1:2 vol/vol). In other experiments tissues were pre-treated with 10 µM U73122 (selective PLC inhibitor) before the addition of ANF or cANP (4–23 amide). Phases were separated by the addition of chloroform and water followed by centrifugation at 2000 *g* for 15 min. The upper phase was applied to an anion exchange column (Bio-Rad X8 resin, 100–200 mesh, formate form) followed by the addition of 5 nM unlabeled myo-inositol. Columns were washed and eluted with 1 M ammonium formate and 0.1 M formic acid. The eluted fraction containing inositol 1,4,5-triphosphate, inositol 1,3,4-phosphate, and inositol 1,2,3,4-tetraphosphate represents PLC activity because inositol 1,4,5-triphosphate ,the immediate product of PLC activity, is the precursor from which the other forms are synthesized [30]. Results were expressed as percentage of control.

2.4. Statistical analysis

Results are expressed as the mean±SEM. The statistical analysis was performed by ANOVA followed by the Student Newman Keuls test. A p of 0.05 or less was considered statistically significant.

3. Results

3.1. Effect of cANP (4–23 amide) on basal and evoked salivation

In the present study ANF as well as the selective agonist of NPR-C receptors, cANP (4–23 amide) were administered into the submandibular gland. Neither the agonist of NPR-C receptors nor ANF elicited

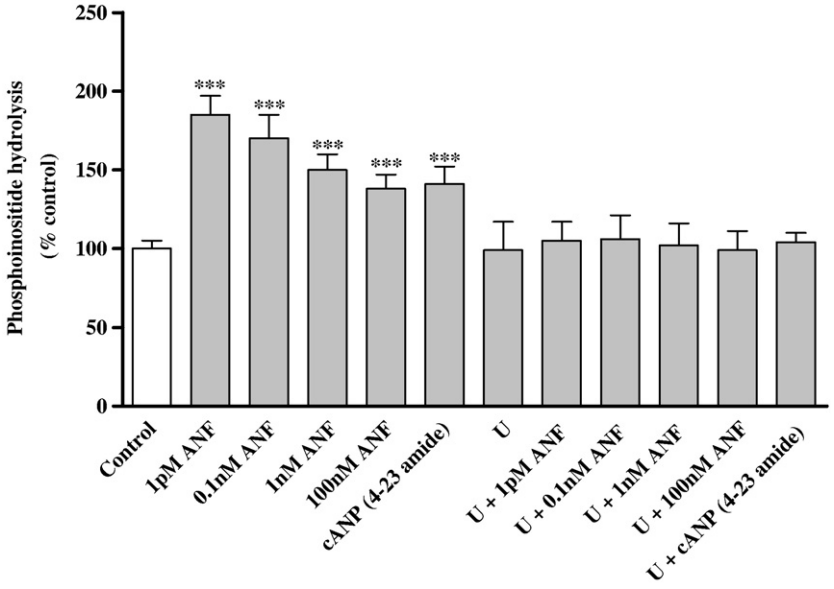

Fig. 2. Effect of ANF and cANP (4–23 amide) on phosphoinositide hydrolysis. Submandibular gland slices were incubated with ANF (1 pM–100 nM) or cANP (4–23 amide) (100 nM) (selective NPR-C agonist) in the presence or absence of U73122 (PLC inhibitor) (U) as described in Materials and methods. Phosphoinositide turnover was determined and expressed as percentage of control. ***: $p<0.001$ vs. control. Number of experiments: 6–8.

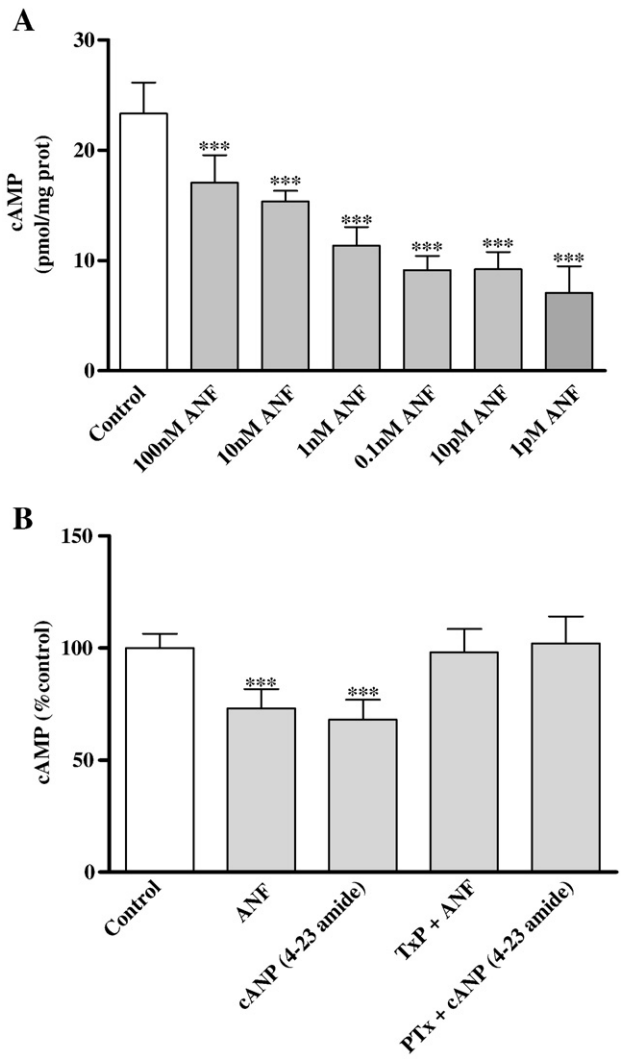

Fig. 3. A) Effect of ANF and cANP (4–23 amide) on basal cAMP intracellular levels in the submandibular gland. Tissue slices were incubated in the presence of ANF (1 pM–100 nM) and cANP (4–23 amide) (100 nM) (NPR-C selective agonist). The content of cAMP was determined and expressed as pmol/mg protein. ***: $p<0.001$ vs. control. Number of experiments: 4–6. B) Effect of ANF on cAMP content in the presence of pertussis toxin (PTx). Submandibular gland slices were pre-treated with PTx as described in Materials and methods and further incubated with 100 nM ANF or 100 nM cANP (4–23 amide). The content of cAMP was determined and expressed as percentage of control. ***: $p<0.001$ vs. control. Number of experiments: 4–6.

salivation *per se* as previously observed when ANF was intravenously applied [15,16].

Stimulated salivation was studied by dose response curves to MC and NE in the absence or in the presence of the selective agonist cANP (4–23 amide) locally administered. The agonist cANP (4–23 amide) potentiated 1, 3 and 10 μg/kg MC-evoked salivation by 111%, 73% and 30%, respectively. When salivation was stimulated by 3, 10 and 30 μg/kg NE, cANP (4–23 amide) increased salivation by 30, 74 and 53%, respectively (Fig. 1A and B). The intraglandular administration of ANF on cholinergic and adrenergic evoked salivary secretion induced similar results (Fig. 1A and B).

3.2. Effect of ANF and cANP (4–23 amide) on PI hydrolysis

ANF dose dependently enhanced PI hydrolysis in the rat submandibular gland. The highest stimulatory effect was achieved with the lowest concentration of the peptide (1 pM). The specific agonist of NPR-C receptors, 4–23 ANP amide, mimicked ANF response supporting that the activation of NPR-C receptors mediated the stimulation of PI turnover induced by the atrial peptide. ANF as well as cANP (4–23 amide) response was abolished in the presence of U-73122, supporting PLC activation mediated by NPR-C receptors (Fig. 2).

3.3. Effect of ANF and cANP (4–23 amide) on cAMP content

The atrial peptide decreased basal cAMP accumulation in the salivary gland in a dose-dependent fashion. The lowest concentration induced the most pronounced decrease in cAMP content (70%) (Fig. 3A). ANF response was mimicked by cANP (4–23 amide) and abolished by pertussis toxin pre-treatment, supporting that the effect was mediated by NPR-C receptors (Fig. 3B). Although ANF decreased basal cAMP levels it did not affect forskolin or isoproterenol-evoked cAMP (Fig. 4A and B). Furthermore, ANF response was inhibited in the presence of U73122 and GF109203X, PLC and PKC inhibitors, respectively but unaltered by KT5023 (PKG inhibitor) (Fig. 5).

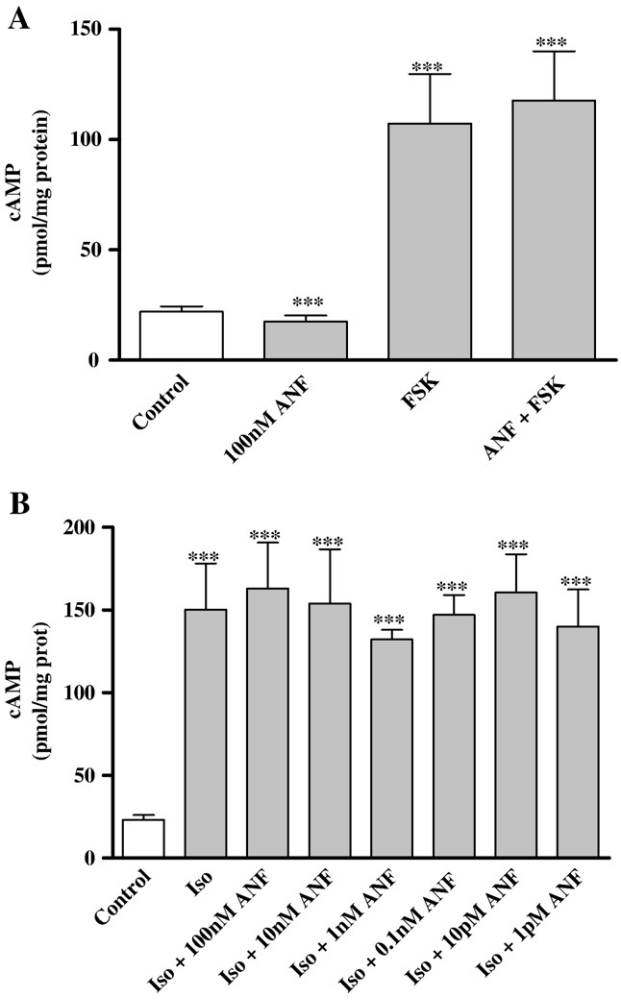

Fig. 4. A) Effect of ANF on forskolin-evoked cAMP. Submandibular gland slices were incubated in the presence forskolin (FSK) (adenylyl cyclase activator) and 100 nM ANF. The content of cAMP was determined and expressed as pmol/mg protein. ***: $p<0.001$ vs. control. Number of experiments: 4–6. B) Effect of ANF and cANP (4–23 amide) on basal isoproterenol-evoked cAMP in the submandibular gland. Tissue slices were incubated in the presence of 100 nM isoproterenol (Iso) and ANF (1 pM–100 nM). The content of cAMP was determined and expressed as pmol/mg protein. ***: $p<0.001$ vs. control. Number of experiments: 4–6.

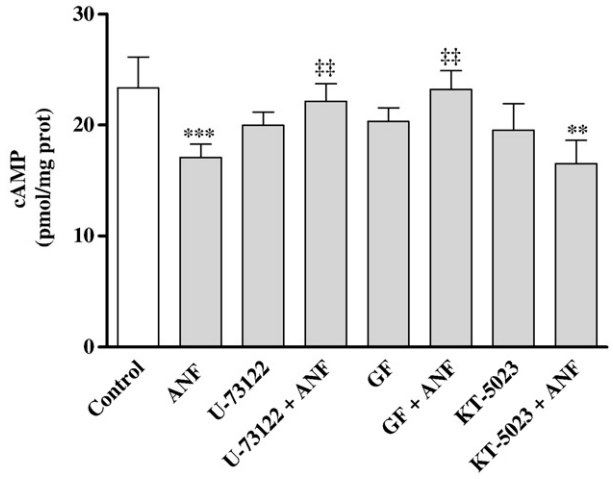

Fig. 5. Effect of PLC, PKC and PKG inhibitors on ANF-evoked reduction of cAMP in the rat submandibular gland. Tissue slices were pre-treated with 10 μM U73122 (PLC inhibitor), GF109203X (GF) (PKC inhibitor) or KT-5023 (PKG inhibitor) and further incubated with 100 nM ANF. The content of cAMP was determined and expressed as pmol/mg protein. **: $p<0.01$ and ***: $p<0.001$ vs. control; ‡‡: $p<0.01$ vs. ANF. Number of experiments: 4–6.

3.4. Effect of ANF and cANP (4–23 amide) on cGMP content

ANF increased the content of cGMP in a dose-dependent fashion, as previously reported. However at the concentration of 1 pM ANF had no effect on cGMP accumulation in the gland, although at that concentration the peptide stimulated PLC and inhibited adenylyl cyclase as shown above. As expected, the selective agonist cANP (4–23 amide) failed to affect cGMP content (Fig. 6).

4. Discussion

We previously reported that ANF is not a sialogogic agonist when systemically applied but it enhances agonist-evoked salivary secretion in the rat. ANF increases muscarinic, α-adrenergic and peptidergic (substance P)-induced salivation but fails to modify that induced by isoproterenol [15,16]. In the present study we investigated the intracellular signalling mechanisms underlying ANF effect in the rat submandibular gland.

Our previous findings supported that ANF response in the submandibular gland was mediated by the activation of non-guanylyl coupled natriuretic peptide receptors since cGMP is not involved in the stimulus-secretion coupling mechanism in the glands [15]. A later study reported the gene expression of ANF and all natriuretic peptide receptors in the gland, suggesting that ANF is a local modulator of salivary secretion [9]. To further confirm this hypothesis in the present study we administered a selective agonist of NPR-C receptors into the submandibular gland and studied basal as well as cholinergic and adrenergic-evoked salivation. The selective agonist is a ring deleted analogue of ANF that interacts specifically with NPR-C [21]. The administration of cANP (4–23 amide) in the gland elicited no secretion neither did ANF, thus confirming that ANF is not a sialogogic agonist. The NPR-C selective agonist potentiated both muscarinic as well as adrenergic stimulated secretion. Similar results were observed in the presence of locally administered ANF, supporting that it enhances cholinergic and adrenergic-evoked salivation through the activation of NPR-C receptors. In previous studies we reported that the intravenous injection of ANF potentiates muscarinic-induced salivation but slightly increases NE-evoked salivary response [15]. However, in the present study ANF intraglandularly administered clearly potentiated not only MC-evoked secretion but also that induced by NE. Nevertheless, the potentiation of the muscarinic salivation induced by ANF or cANP (4–23 amide) was more pronounced (i.e. at 3 μg/kg/h MC or NE the percentage of increase in the presence of cANP (4–23 amide) was 73 and 30% respectively).

A natriuretic peptide system was described in the rat submandibular gland [9]. The authors reported the presence of mRNA ANF and C-type natriuretic peptide as well as those of the guanylyl coupled and uncoupled natriuretic peptide receptors (NPR-A, NPR-B and NPR-C). Furthermore, they also showed the existence of ANF prohormone in the glands. These findings provide strong evidence of local synthesis of the natriuretic peptides and possible paracrine and/or autacrine effects on the gland functionality when locally released. This may partially explain the difference found when ANF was given through different routes of administration; the systemic infusion of the peptide induced a less potent agonist-evoked secretory response than when given intraglandularly [15]. In addition, the metabolization of ANF when intravenously applied is another factor that may eventually affect the magnitude of the response.

The stimulatory effect on evoked salivary secretion by ANF was mediated by the NPR-C receptor since ANF response was mimicked by cANP (4–23 amide), selective agonist of NPR-C receptor. This receptor subtype is structurally different from the GC coupled receptors (NPR-A and NPR-B). The intracellular domain of NPR-C consists of only 37 amino acids and is devoid of GC and kinase activities [21]. This receptor subtype is a G-protein coupled receptor that through Gi couples to the activation of PLC and the inhibition of adenylyl cyclase [21–25]. Although NPR-C receptors are widely distributed in different organs and cell types, in the submandibular gland its density is relatively high as compared to those of the other natriuretic peptide receptors [9]. Furthermore the expression of NPR-C is only two fold less than that found in the lungs, an organ known for its abundant expression of this receptor subtype [9]. The NPR-C receptor also mediates natriuretic peptide effects on pancreatic and bile secretions in the rat [31–33].

When the intracellular signalling pathways coupled to the NPR-C receptor were investigated in the submandibular gland, it was found that ANF dose dependently enhanced PI turnover and that this effect was abolished in the presence of U-73122 supporting PLC activation by the atrial peptide. Furthermore, ANF response on PI hydrolysis was mimicked by the selective agonist of NPR-C receptors. These findings suggest that the stimulatory effect of ANF on the salivation evoked by sialogogic agonists that signal through PLC activation is likely attributed to the fact that the agonists as well as ANF share the same intracellular signalling pathway, although coupled to different G

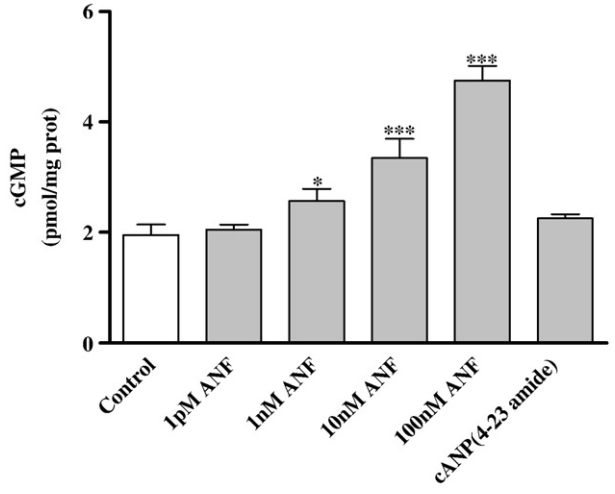

Fig. 6. Effect of ANF and cANP (4–23 amide) on basal cGMP. Submandibular gland slices were incubated the presence of ANF (1 pM–100 nM) or cANP (4–23 amide) (4–23 ANP) (100 nM). The content of cGMP was determined and expressed as pmol/mg protein. *: $p<0.05$, and ***: $p<.001$ vs. control. Number of experiments: 5–7.

proteins. The lowest concentration of ANF (1 pM) evoked the highest stimulatory effect on PI turnover. This finding is probably related to the high density of NPR-C receptors present in the gland. ANF also stimulates PLC through NPR-C receptors in the exocrine pancreas and other cell types [12,17,25,33,34]. The observation that cANP (4–23 amide) increased PI turnover supports that the response is not mediated by NPR-A or NPR-B receptors and resultant increase in cGMP because the selective agonist is ineffective in altering cGMP levels as shown in the present and previous studies [35]. Furthermore it was shown that ablation of NPR-C receptors by NPR-C receptor antisense inhibits cANP (4–23 amide)-mediated stimulation of PI hydrolysis [35].

The atrial peptide also dose dependently reduced basal cAMP levels in the submandibular gland through NPR-C activation. The response was mimicked by the selective agonist of NPR-C receptors, cANP (4–23 amide) and abolished by pertussis toxin pre-treatment supporting Gi activation consistent with previous studies in other tissues and cell types [19,22–25]. The lowest concentration of ANF (1 pM) evoked the highest inhibitory effect showing a correlation with PLC activation. Although ANF reduced the basal content of the cyclic nucleotide it failed to modify isoproterenol-evoked cAMP. These findings suggest that ANF through NPR-C receptors inhibited the basal activity of the enzyme but not that stimulated by the β-adrenergic agonist. This is consistent with the observation that the intravenous administration of ANF does not affect isoproterenol-evoked salivary flow in the submandibular gland [15]. However it enhances isoproterenol-evoked amylase release in the parotid gland, likely through NPR-C receptors coupled to PLC activation and ultimate intracellular calcium increase [17]. The reduction in basal cAMP produced by ANF was abolished by PLC and PKC inhibitors supporting that the response was mediated by the PI pathway. However, the decrease in cAMP was not modified by a PKG inhibitor supporting that natriuretic peptide GC coupled receptors were not involved in the inhibitory effect of ANF on the adenylyl cyclase/cAMP system in the gland.

A previous study showed that ANF increases PI turnover and decreases cAMP accumulation in vascular smooth muscle cells [34]. The authors proposed that in this cell type the increase in PI hydrolysis was the resultant of adenylyl cyclase inhibition. In the present study the reduction of cAMP content induced by ANF was abolished by PLC and PKC inhibitor supporting that this intracellular signalling pathway was involved in adenylyl cyclase inhibition. Consistent with present findings it was shown that PKC activation is involved in attenuation of adenylyl cyclase inhibition by ANF [36]. A role of PKC in the phosphorylation of Gi proteins and resultant uncoupling of receptor from adenylyl cyclase has been reported [37]. However the role of PKC on adenylyl cyclase regulation is still rather controversial likely due to the differential regulatory mechanisms underlying the different isoforms of the enzyme [38]. In the present study the stimulation of PI turnover by NPR-C activation may be likely mediated by the release of βγ subunits of inhibitory G proteins which has been shown to stimulate PLC [39].

The correlation between PLC activation and cAMP inhibition at the different concentrations of ANF appears at first sight intriguing provided that the lowest concentrations of the peptide evoked the most marked effect on the intracellular signalling coupled to the receptor. As evidence supports that ANF is locally synthesized in the submandibular gland, it is likely that low concentrations locally released are sufficient to trigger the intracellular signalling involved in the modulation of evoked salivary secretion. Higher concentrations of the peptide in the presence of agonists would probably saturate the secretory response.

Both calcium-mobilizing and cAMP-generating signalling pathways have been linked to salivation [1]. An increase in intracellular calcium is the primary fluid secretion signal in salivary acinar cells. In contrast, cAMP signals regulate secretory granule discharge but produce little fluid on their own [1]. The sympathetic and parasympathetic nervous system act synergically enhancing salivary secretion through the stimulation of PLC, mediated by muscarinic and α-adrenergic receptors, and adenylyl cyclase mediated by β-adrenergic receptors. Although activation of calcium-dependent ion channels is the primary mechanism underlying fluid secretion, salivation can be significantly enhanced when both calcium and cAMP signalling systems are activated concurrently [1,40]. ANF fails to induce salivation *per se* but enhances both muscarinic and adrenergic-evoked salivation. ANF is not a sialogogic agonist likely due to the fact that although it stimulated PLC, it also decreased cAMP content. The interaction between the two signalling pathways triggered by ANF may probably result in a sub-threshold stimulus to induce salivation.

The content of cGMP was increased by ANF in a dose-dependent manner as previously reported by other authors supporting activation of guanylyl cyclase coupled receptors [41]. However ANF at 1 pM failed to modify cGMP content whereas at that concentration it evoked inhibition of adenylyl cyclase and stimulation of PLC. The activation of NPR-A and NPR-B receptors are likely to mediate functions others than salivary secretion because cGMP is not involved in the stimulus-secretion coupling mechanism in the submandibular gland [1]. Diverse studies have implicated cGMP in the stimulation of capacitative or store-operated calcium entry which is activated following the emptying of agonist-sensitive intracellular calcium stores [42]. However the regulation of the activity of the store-operated calcium channels in salivary glands remains poorly understood [1].

This study shows that the selective agonist of NPR-C receptors, cANP (4–23 amide) intraglandularly administered induced no salivation but enhanced muscarinic and adrenergic-evoked salivary secretion similarly to ANF. These findings support that ANF modulates agonist-induced salivation in the submandibular gland through the activation of NPR-C receptors coupled to PLC activation and adenylyl cyclase inhibition, and further confirm that ANF is a local modulator of cholinergic and adrenergic-evoked salivary secretion in the rat.

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