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Atrial Natriuretic Factor-Induced Amylase Output in the Rat Parotid Gland Appears to be Mediated by the Inositol Phosphate Pathway

L. G. Bianciotti,† M. S. Vatta,* J. C. Elverdin,§ M. B. di Carlo,‡ G. Negri,‡ and B. E. Fernandez†
**Cátedras de Fisiología, †Cátedras de Fisiopatología, y ‡División Gastroenzimología del Departamento de Análisis Clínicos. Facultad de Farmacia y Bioquímica, y §Cátedra de Fisiologí, Facultad de Odontología, Universidad de Buenos Aires, Argentina*

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In previous *in vivo* studies we have reported that atrial natriuretic factor enhanced induced salivary secretion and increased isoproterenol-induced amylase release in the rat suggesting that, ANF effect could be mediated by phosphatidylinositol hydrolysis. In the present work, the effect of ANF on rat parotid tissue incubated *in vitro* was investigated with the aim to assess whether the phosphoinositol pathway was involved in ANF intracellular signaling in the parotid gland. Results showed that ANF induced a dose dependent increase in amylase fractional release, which was lower than that evoked by any concentration of isoproterenol. Furthermore 100 nM ANF enhanced isoproterenol-evoked amylase release. The effect of ANF was not affected in the presence of propranolol suggesting the noninvolvement of the β adrenergic receptor, which is the main stimulus for the output of the enzyme in the parotid gland. However, ANF increased phosphatidylinositol hydrolysis, which implies an increase in intracellular calcium, which is necessary for the achievement of maximal response in amylase release. This effect was abolished in the presence of neomycin supporting ANF direct stimulation of phospholipase C. These results suggest the involvement of the C type natriuretic peptide receptor coupled to phospholipase C in ANF evoked amylase release and ANF enhancement of the isoproterenol-induced output of the enzyme.

Atrial natriuretic factor (ANF) is a 28 aminoacid peptide which is synthetised by and stored in the atrial myocytes. It is released in response to atrial stretch, endothelin 1 and α1-adrenergic stimulation (1). This hormone exhibits potent diuretic, natriuretic and vascular smooth muscle relaxant properties. Furthermore, ANF is also involved in the maintenance of water and salt homeostasis (1).

Increasing evidence suggests the role of ANF as a modulator of the biological responses of the digestive system. ANF modulates biliary, intestinal, pancreatic and salivary secretions as well as bowel motility in the rat (2–5). Although cGMP is the intracellular messenger that mediates most of ANF biological effects, this second messenger seems not to be involved in the effects of ANF on the digestive exocrine glands, in spite of the finding that cGMP increases have been reported to occur upon ANF receptor stimulation in the pancreas as well as the salivary glands (6,7). The finding of mRNA ANF in the gastrointestinal tract and the digestive exocrine glands suggests local synthesis of the peptide and supports a paracrine role (8–10).

We have previously reported that ANF was not a sialogogic agonist at least in the concentrations tested, although it enhanced the salivary secretion induced by different sialogogic agonists in rat. ANF potentiated metacholine, methoxamine and substance P evoked salivation both in the parotid and submaxillary glands (4). Furthermore, ANF also modified the electrolyte excretion pattern induced by these sialogogic agonists (11). Coversely, when salivation was evoked by a β-adrenergic agonist such as isoproterenol, ANF did not modify the salivary flow, however it potentiated protein and amylase induced output (4,11).

Most of ANF biological effects are mediated by ANF receptors coupled to guanylate cyclase however in the salivary glands cGMP is neither physiologically involved in primary saliva formation nor in amylase output (12).

Stimulus secretion coupling in the salivary glands is mediated by receptor activation (muscarinic, adrenergic o peptidergic) on the basolateral membrane which leads to phosphoinositide hydrolysis and inositol triphosphate (IP3) and dialcylglicerol (DAG) generation through the activation of phospholipase C (12). IP3 formation results in an increase in cytoplasmic calcium

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and the loss of water and electrolytes from the acini to for the primary saliva (12,13). β-adrenergic stimulation is involved in protein and amylase output, although cytoplasmic calcium increase appears to be important for the achievement of maximum response (12,14).

On this basis the aim of the present work was to assess whether the PI pathway was the intracellular mechanism that mediates ANF effect in the parotid gland.

MATERIALS AND METHODS

Wistar strain male rats (Facultad de Farmacia y Bioquímica, University of Buenos Aires) weighing 250–300 g were used in the experiments. Animals were housed in group cages under conditions of controlled temperature (22 ± 1°C) and illumination (12hs light/dark cycle) and maintained *ad libitum* on Purina Laboratory chow and tap water. The animals were deprived of food, but allowed free access to water, 14 hs before the experiments in order to avoid modifications in the digestive hormones and peptides which could eventually alter the different digestive secretions. Rats were killed by cervical dislocation and parotid glands quickly removed and placed on ice on a Petri dish containing gassed bicarbonate-Krebs solution of the following composition (in mM): NaCl 118; Kcl 4.7; $MgCl_2$ 1.2; NaH_2PO_4 1.0; $CaCl_2$ 2.5; EDTA-Na 0.004; dextrose 11.1; $NaHCO_3$ 25.0; ascorbic acid 0.11. Extraglandular tissue as well as the main excretory duct were removed under microscope. Parotid tissue was cut and preincubated for 5 min and then incubated for 20 min with Krebs, pH 7.4, at 37°C and gassed with a gaseous mixture of 95% O_2 plus 5% CO_2.

Amylase output was studied in the presence of different concentrations of isoproterenol (IP) (Sigma, Chem. Co., MI, USA), ANF (Peninsula Lab., Belmont, CA, USA) and 8Br cGMP (Sigma Chem. Co., MI, USA). To investigate the possible involvement of β adrenoceptors as mediators of ANF response, amylase release was also studied in the presence of propranolol (PP) (Sigma Chem. Co., MI, USA).

The following groups were studied: Control (no drugs added); 1, 10 and 100 nM ANF alone or in the presence of 1 μM IP or 0.1 PP; 0.01, 01 and 1 μM IP; 0.1, 1 and 10 μM 8Br cGMP.

Incubation media were centrifuged and supernatants saved for amylase determination. Tissues were homogenized, centrifuged and supernatants saved for amylase determination. Tissues were homogenized, centrifuged and supernatants saved for amylase determination. Amylase activity was assessed in diluted samples by an enzymatic-colorimetric test (Boeheringer-Mannheim GmbH) using α-4-nitrophenylmaltoheptaoside as substrate (15). Amylase activity is expressed as fractional release (amylase activity in the incubation media/amylase activity in the incubation media plus tissue × 100).

Phosphoinositide hydrolysis was determined according to the method described by Berridge et al (16). Briefly, parotid glands were removed, placed on ice in a Petri dish containing Krebs solution and chopped. Tissues were preincubated for 5 min in 500 μl of Krebs-bicarbonate solution, pH 7.4 and gassed with a mixture of 95% O_2 + 5% CO_2 for 5 min and then incubated with 500 μl of Krebs-bicarbonate solution/10nM LiCl containing 2 μCi/500 μl of myo [$^{2\text{-}3}$H]-inositol for 2 hs. Thirty minutes before the end of the incubation period, ANF and/or the other drugs were added to the medium. Tissues were then washed twice with fresh cold Krebs solution for 5 min and homogenized with chloroform:methanol (1:2 v/v). To separate the phases, 620 μl chloroform and 1 ml of water were added to the homogenates which were then centrifuged at 2000 g for 15 min. The upper phase was applied to an anion exchange column (Bio-Rad X8 resin, 100-200 mesh, formiate form) followed by the addition of 5 mM myo-inositol. Columns were then eluted with 1M amonium formiate and 0.1 formiate acid. The eluate fraction contains the isomers of 1,4,5 inositol triphosphate, 1,3.4. inositol phosphate as well as 1,2,3,4 inositol tetraphosphate. This fraction represents phospholipase C activity since 1,4,5 inositol triphosphate, the immediate product of phospholipase C activity is the precursor from which the other forms are synthetized (17). Radioactivity was determined by usual scintillation counting methods. Results are expressed as percentage of control value.

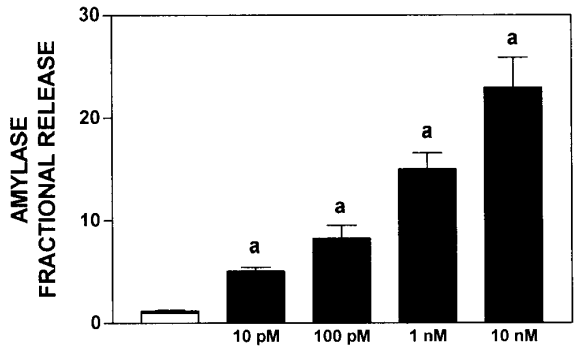

FIG. 1. Effects of isoproterenol (IP) on amylase fractional release. Open column: Control group; solid column: groups incubated with different concentrations of IP. a: $p < 0.001$ as compared with control group. Data is shown as the mean ± S.E.M.; Number of experiments: 6–8.

Amylase and PI hydrolysis results are expressed as the mean ± S.E.M. One way ANOVA and the t test modified by Bonferroni were used for statistical analysis. A p of 0.05 or less was considered statistically significant.

RESULTS

Figure 1 illustrates the effect of the β-adrenergic agonist IP on amylase fractional release. This agonist increased amylase release in a dose dependent manner as it is widely described in the literature. Furthermore, the ability of the parotid tissue to release amylase in response to IP stimulation supports the integrity and viability of the preparation.

We have previously reported that ANF enhanced IP-induced amylase release *in vivo* (11). In the present work we studied the effects of ANF on amylase release in parotid tissue incubated *in vitro*. Results showed that 1, 10 and 100 nM ANF increased amylase fractional output. The stimulant effect induced by ANF was dose dependent but lower than that evoked by any concentration of IP (Fig. 2).

As the β-adrenoceptors activation is the main stimuli for amylase release in the parotid gland, we assessed whether ANF stimulated-output involved the activation of this receptor. Tissues were incubated in the presence of both ANF and PP. Results showed that the enhancement of amylase release evoked by the natriuretic factor (100 nM) was not inhibited by 0.1 μM PP (which is the concentration reported to inhibit amylase release induced by 1 μM IP) (Fig. 3), which suggests the non involvement of the adenylate cyclase/cAMP system in ANF intracellular signaling in the parotid gland.

In order to investigate whether the enhancement of

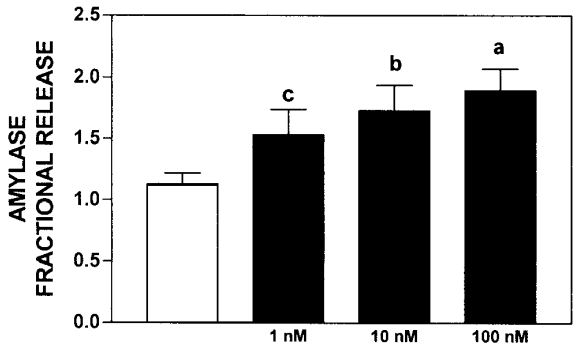

FIG. 2. Effects of atrial natriuretic factor (ANF) on amylase fractional release. Open column: control group; solid column: groups incubated with different concentrations of ANF. a, b and c: $p < 0.001$, 0.01 and 0.05 respectively as compared with control group. Data is shown as the mean ± S.E.M.; Number of experiments: 8–12.

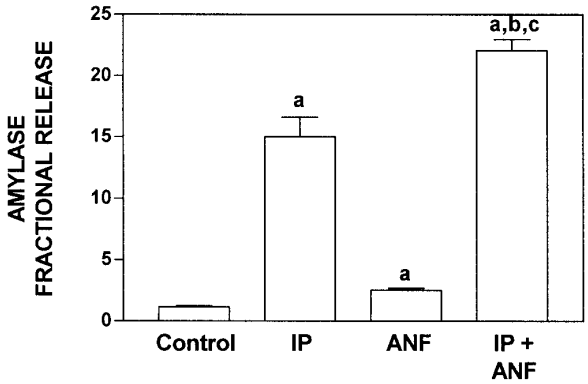

FIG. 4. Effects of atrial natriuretic factor (ANF) on amylase fractional release in the presence of isoproterenol (IP). a: $p < 0.001$ as compared with the control group; b: $p < 0.005$ as compared with 1 μM IP group; c: $p < 0.001$ as compared with 100 nM ANF group. Data is shown as the mean ± S.E.M.. Number of experiments: 5–8.

IP induced amylase release evoked by ANF, as previously reported, also occurred *in vitro*, parotid tissue was incubated with IP + ANF. Results showed that 1 and 10 nM ANF did not modify 1 μM IP-induced amylase output (data not shown). However, 100 nM ANF enhanced the β-adrenergic stimulated release of the enzyme (Fig. 4), which is in accordance with previous findings (11).

As cGMP is the intracellular messenger that mediates most of ANF biological effects reported in different cell types, amylase release was also studied in the presence of different concentrations of the permeable analogue of c GMP. Results showed that 1, 10 μM 8 Br-GMPc increased amylase output while 100 nM 8Br cGMP had no effect on the enzyme release (Fig. 5).

Phosphoinositide turnover was studied in order to assess whether ANF effect in the parotid gland could be mediated by the activation of phospholipase C activity which is a key enzyme in the stimulus-secretion coupling in the salivary glands. Results indicated that 1, 10 and 100 nM ANF stimulated PI hydrolysis in the parotid gland (Fig. 6). Furthermore, the enhancement of PI hydrolysis evoked by ANF was inhibited in the presence of neomycin, suggesting that ANF stimulates phospholipase C activity in a direct way (Fig. 7).

DISCUSSION

In previous works we demonstrated that ANF enhanced the salivary secretion induced by sialogogic agonists (metacholine, methoxamine and substance P) which stimulate PI hydrolysis in both the parotid and submaxillary glands of the rat (4). Furthermore, in the submaxillary gland, ANF also modified the excretion pattern of sodium and potassium induce by these sialogogic agonists, suggesting thus an effect of the atrial factor not only at the acinar but also at the ductal level of the salivary glands (11). When salivation was evoked by a β-adrenergic agonist such as IP, ANF did not mod-

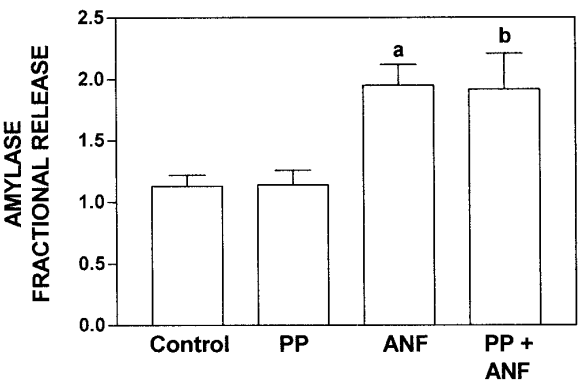

FIG. 3. Effects of atrial natriuretic factor (ANF) on amylase fractional release in the presence of propranolol (PP). a: $p < 0.001$ as compared with control group; b: $p < 0.05$ as compared with PP group. Data is shown as the mean ± S.E.M. Number of experiments: 5–8.

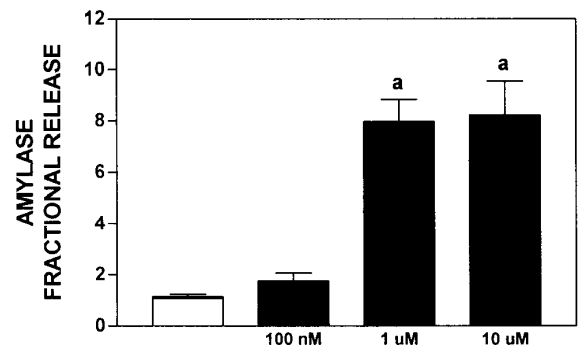

FIG. 5. Effects of 8Br cGMP on amylase fractional release. Open column: control group; solid column: groups incubated with different concentrations of 8Br cGMP. a: $p < 0.001$ as compared with the control group. Data is shown as the mean ± S.E.M. Number of experiments: 6–9.

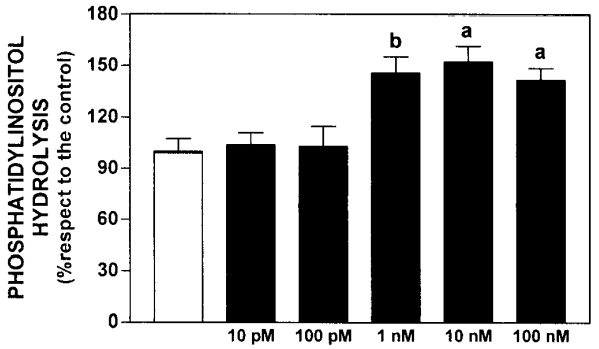

FIG. 6. Atrial natriuretic factor (ANF) effect on phosphatidylinositol hydrolysis. Open column: control group; solid column: groups incubated with different concentrations of ANF. a and b: $p < 0.001$ and 0.005 as compared with the control group. Data is shown as the mean ± S.E.M. Number of experiments: 5–9.

ify the volume of saliva secreted, but enhanced the excretion of total proteins and amylase from the parotid gland (4,11). The present results confirm the latter finding, since 100 nM ANF potentiated the release of amylase evoked by IP. The other concentrations of ANF tested did not modified the evoked output of the enzyme. As IP induces a marked increase in amylase release as compared with that evoked by ANF, it is possible that the effect of 1 and 10 nM ANF is overlooked by the effect of IP.

On the other hand, 1, 10 and 100 nM ANF increased amylase fractional release in a dose dependent fashion. The latter finding has not been observed in previous in vivo experiments, since the atrial factor failed to elicit salivation *per se* (4). However, ANF enhancement of amylase output was always lower than that evoked by any of the concentrations of IP used, which suggests a modulatory role for ANF in the output of the enzyme.

Cyclic AMP and PI hydrolysis are two signal transduction pathways that frequently act in concert in many endocrine as well as exocrine cells for the release of hormones or enzymes (18). In the salivary glands the stimulus secretion coupling for the formation of primary saliva involves the activation of phospholipase C which generates two second messengers: IP3 and DAG. IP3 increases intracellular calcium while DAG activates protein kinase C which is involved in the phosphorilation of different substrates that participate of the exocytosis mechanism. The mobilization of calcium to the cytoplasm triggers the loss of electrolyte and water from the acinar cells to for the primary saliva, which is later modified within the duct system (12,13). On the other hand, cAMP in the salivary glands, as well as in other exocrine glands, is associated with the excretion of organic compounds such as enzymes (12).

In most tissues the biological effects of ANF are mediated by intracellular accumulation of cGMP through the activation of the biological receptors coupled to guanylate cyclase (19). Molecular cloning studies have revealed the existence of three types of natriuretic peptides receptors. Types A and B receptors (ANFR-A and ANFR-B) are coupled to guanylate cyclase whereas the type C receptor (ANFR-C) or clearance receptor was initially shown to be silent (19). However, there is evidence that this receptor is coupled to other second messenger system as adenylate cyclase/cAMP and phospholipase C/IP3-DAG (19).

In the salivary glands ANF receptors have been reported as well as the increase in cGMP upon receptor stimulation (7,10). However, as it was previously mentioned adenylate cyclase and phospholipase C products are the second messenger systems involved in the physiological activation of the secretory response in the salivary glands. ANF increased amylase output from parotid slices and furthermore, it also enhanced the release of the enzyme evoked by IP. ANF evoked amylase release was not inhibited by PP, which suggests that the β-adrenoceptor is not involved in the output of amylase induced by the atrial factor and that cAMP is not the intracellular signal that mediates ANF effect in the parotid gland, which is consistent with the literature that indicates that ANF does not stimulate cAMP generation. In fact, the inhibition of adenylate cyclase induced by ANF has been demonstrated in several tissues (19).

As 8 Br-cGMP increased amylase output in a dose dependent manner it can be argued that cGMP could be the intracellular messenger that mediates ANF stimulated amylase output in the parotid gland. However, cGMP does not seem to be physiologically involved in amylase release in this gland (12). Nevertheless, several authors have reported increases in amylase release following the stimulation with permeable analogues of cGMP (12). It has been demonstrated that

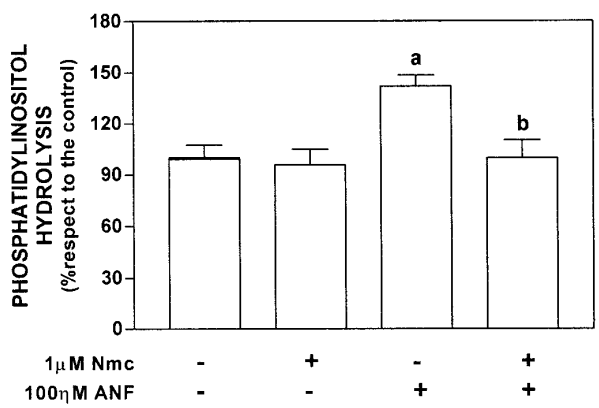

FIG. 7. Effects of atrial natriuretic factor (ANF) on phosphatidylinositol hydrolysis in the presence of neomycin (Nmc). a: $p < 0.001$ as compared with the control group; b: $p < 0.01$ as compared with ANF group. Data is shown as the mean ± S.E.M. Number of experiments: 5–9.

cGMP stimulates amylase release from mouse parotid glands and further, it may be required for the muscarinic augmentation of amylase release by forskoline (20). Moreover, cGMP has also been shown to increase calcium uptake into the parotid acini, which may be related with the exocytotic release of the enzyme. It was suggested that guanylate cyclase may interact with protein kinase C or calcium. In fact protein kinase C and calcium have been shown to directly stimulate gualylate cyclase activity (21). Signal induced inositol phospholipid breakdown is usually associated with arachidonic acid release and cGMP formation. Protein kinase C, calcium mobilization, arachidonic acid release and cGMP formation appear to be integrated in a single cascade. Increasing evidence suggests that cGMP may act as a negative rather than a positive mediator providing an intracellular feedback control to prevent over-response (22). It has been shown that 8 Br-cGMP inhibits signal induced phosphoinositide breakdown and thereby counteracts the activation of protein kinase C and may also take part in the decrease of intracellular calcium (18). However information on the role of this cycle nucleotide in secretory tissues is still limited. If evidence supports the role of cGMP as a negative feedback to avoid over-response, it is not clear why it induces amylase release in the parotid gland. Although cGMP derivatives facilitate enzyme release, their effect is not probably physiological. This issue has been well studied in the pancreas (22). In this tissue the increase in amylase output in the presence of cGMP analogues has been reported. However it has been suggested that it might result from the ability of cGMP analogues to mimick cAMP or to induce cAMP accumulation (22,23). Other authors indicated that it appears that cGMP is formed not as direct result of receptor occupation but as a secondary response to other receptor activated events and it has been proposed that its rate of formation is normally increased by stimulus-evoked increases in cytosolic free calcium (12). Present results seem to support this hypothesis since ANF increased PI hydrolysis which results in an increase of intracellular calcium. Therefore, cGMP does not appear to be a direct link between calcium and enzyme release although its increased generation is apparently mediated by calcium. In addition, it has been reported that the agonists that increase intracellular calcium also increase cGMP generation (12). Moreover there is still no compelling evidence to suggest that cGMP plays a role in the stimulus secretion coupling in salivary glands (12). So far it can be concluded that although cGMP is increased in the presence of ANF and the permeable analogue facilitates amylase release, cGMP seems not to mediate ANF intracellular signal for amylase release. The increase in cGMP may also be related to other physiological events within the acinar cells.

A great body of evidence supports that amylase release is dependent upon cAMP accumulation through the activation of β-adrenoceptors (12). However, maximal amylase or protein secretion in the parotid gland requires both cAMP and calcium. Moreover, increases in intracellular calcium are known to activate adenylate cyclase via calmodulin in the parotid gland (24). ANF inhibits adenylate cyclase in several cell types and tissues through the inhibitory guanine nucleotide regulatory protein (Gi) coupled effector pathway (19). ANF inhibition of adenylate cyclase in the parotid gland has been reported by Shinomura et al. (25) who also showed that the atrial peptide diminished amylase release. There is an obvious discrepancy between the latter observation and the present results, however our results are consistent with previous *in vivo* experiments in the rat (11).

In view that ANF increased amylase fractional release but cAMP seemed not to mediate the intracellular signalling, another possibility was that ANF might increase intracellular calcium through the activation of phospholipase C. Therefore we studied the effect of ANF on PI turnover. Results showed that ANF stimulates PI hydrolysis in the rat parotid gland, which suggests that the atrial factor in involved in the stimulus secretion coupling in this gland. This observation supports previous findings in which ANF enhanced the salivary response evoked by sialogogic agonists that signal through the PI pathway (4). Furthermore it also supports ANF enhancement of amylase release induced by IP, since increased intracellular calcium is important for the achievement of maximal secretion of proteins in several exocrine glands including the parotid gland (26). Furthermore the ANF effect on PI hydrolysis was abolished by neomycin which inhibits phospholipase C activity. This results suggest that ANF stimulates phospholipase C activity.

Present data supports that the effect of ANF on the parotid gland is likely to be mediated by the ANFR-C receptor which has been associated with the inhibition of adenylate cyclase and the stimulation of PI hydrolysis in several cell types (19).

In conclusion, ANF stimulated spontaneous and IP evoked amylase fractional release in the parotid gland as well as PI hydrolysis, suggesting that this second messenger system mediates, likely upon the type C receptor (ANFR-C) stimulation, its effect in the parotid gland of the rat. The fact that ANF does not induce salivation *per se* and that its mRNA has been demonstrated in the parotid glands, supports the role of this peptide as a modulator of the secretory response by acting probably in a paracrine or autacrine manner.

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