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Effect of SNI-2011 on amylase secretion from parotid tissue in rats and in neuronal nitric oxide synthase knockout mice

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Abstract

The effect of (±)*cis*-2-methylspilo(1,3-oxathiolane-5,3′)quinuclidine (SNI-2011) on the secretory pathway of amylase in parotid tissues was investigated. SNI-2011-induced exocytosis was inhibited by a cell-permeable Ca^{2+} chelator or inhibitors of calmodulin kinase II, neuronal nitric oxide synthase (nNOS), soluble guanyl cyclase, cyclic GMP-dependent protein kinase (PKG), and myosin light chain kinase, suggesting that these enzymes were coupled with the exocytosis. Stimulation with SNI-2011 of isolated rat parotid acinar cells loaded with 4,5-diaminofluorescein/diacetate (DAF-2/DA) induced a fast increase in DAF fluorescence corresponding to an increase in the NO production. SNI-2011-induced amylase secretion from parotid tissues in nNOS knockout mice has not been observed yet in spite of the expression of muscarinic M_3 receptors and the maintenance of secretory response to isoproterenol in the tissues. These results indicate the implication of the activation of Ca^{2+}- and calmodulin-dependent enzymes and NOS-PKG signaling pathway in SNI-2011-induced amylase secretion from parotid acinar cells.

Keywords: SNI-2011; Amylase secretion; Nitric oxide (NO) synthase; NOS knockout, mouse, neuronal; Guanyl cyclase, soluble; Myosin light chain kinase

1. Introduction

Xerostomia is characterized by oral dryness and difficulty both in performing oral functions, such as eating, speaking, and swallowing, as well as in tolerating dentures. This condition results from an impairment in fluid secretion by the salivary glands, and medical treatments that induce long-lasting salivary secretion have been sought as a cure. The drug (±)*cis*-2-methylspilo(1,3-oxathiolane-5,3′)quinuclidine (SNI-2011), which is a structurally rigid analog of acetylcholine, induces long-lasting secretion of saliva in rats and dogs (Masunaga et al., 1997). We have previously shown that SNI-2011, through interaction with muscarinic M_3 receptors, induces a persistent increase in the amount of aquaporin-5 in the apical plasma membrane of rat parotid acinar cells, an effect that appears to be mediated by a long-lasting increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ishikawa et al., 2000). Exocytosis in parotid acinar cells stimulated by muscarinic M_3 receptor agonists is also associated with an increase in $[Ca^{2+}]_i$ (Foskett et al., 1991), suggesting that SNI-2011 may induce amylase secretion from these cells.

The Ca^{2+} binding protein calmodulin plays an important role in Ca^{2+}-mediated signal transduction. The Ca^{2+}–calmodulin complex activates several protein kinases as well as other enzymes. Five Ca^{2+}- and calmodulin-dependent enzymes have been identified in the pancreas: calmodulin kinase I, II, and IV as well as myosin light chain kinase (MLCK) and nitric oxide synthase (NOS) (Mizuno et al., 2000; Wang et al., 1994; Yule and Williams, 1994). Calmodulin kinase II is a multifunctional enzyme that is required both for secretory granule mobilization under stimulated conditions and for maintenance of secretory capacity under basal conditions in pancreatic β cells (Gromada et al., 1999). However, it remains unclear which calmodulin kinases have function in enzyme secretion from pancreatic and parotid acinar cells. MLCK plays an important role in stimulus–secretion coupling in several secretory cells. MLCK appears to function in Ca^{2+}-dependent secretion, given that the secretion of both insulin (Iida et al., 1997) and catecholamines (Kumakura et al., 1994) is inhibited by a selective inhibitor, ML-9, of this kinase. In addition, MLCK is thought

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to regulate capacitative Ca^{2+} entry into cells after the depletion of intracellular Ca^{2+} stores (Watanabe et al., 1998). MLCK activity is also responsible for the increase in the extent of phosphorylation of myosin light chain (MLC) induced by cholecystokinin in rat and mouse pancreatic acini (Iida et al., 1997). However, the role of MLCK in the secretory machinery of parotid acinar cells remains unknown.

It has well been known that nitric oxide (NO) is produced by three isoforms of NOS, namely endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) in mammalian tissues. Localization of nNOS in rat salivary glands was immunoreactively shown by Lohinai et al. (1997). It has also reported that NO generation through activation of muscarinic receptors in rat parotid acinar cells, rat submandibular gland cells, and rat pancreatic acinar cells is regulated calcium-dependently (Merrit and Rink, 1987; Xu et al., 1997; Sugiya et al., 2001).

The actions of NO generated by NOS in signaling pathways are mediated predominantly by cyclic GMP and cyclic GMP-dependent protein kinase (PKG) (Clementi, 1998). Recently, we reported that nNOS was endogenously present in rat parotid acinar cells and that the rapid activation of this enzyme together with those of calmodulin kinase II and PKG contributed to methacholine-induced amylase secretion (Ishikawa et al., 2002a) and acetylcholine-induced the increase in the amount of aquaporin-5 in the apical plasma membrane (Ishikawa et al., 2002b). However, it has not been clear whether SNI-2011 regulates NOS-PKG signaling in rat parotid cells. We have now demonstrated by using Western blot analysis the expression of nNOS in isolated parotid acinar cells of rats. We have also shown that SNI-2011 stimulated amylase secretion and, with the use of the fluorescent NO indicator DAF-2/DA, that SNI-2011 induced a marked increase in DAF-2 fluorescence in these cells. It has also shown in this study that parotid tissues from nNOS knockout mice did not show amylase secretory response to SNI-2011 in spite of the expression of muscarinic M_3 receptors and the maintenance of secretory response to isoproterenol in the tissues. Finally, our results suggest that Ca^{2+}–calmodulin-dependent enzymes, nNOS, calmodulin kinase II, MLCK, and PKG contribute to the induction of amylase secretion by SNI-2011.

2. Materials and methods

2.1. Animals and diet

Male Wistar rats (8 weeks old) were used for the experiments. Wild-type (male, B6; 129SF2/J101045) and mutant mice lacking nNOS (male, B6; 129S-NOS 1^{tm1plh}), weighing 18–23 g, were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and used for some experiments. They were provided with a standard laboratory diet (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum, and were maintained in a temperature-controlled environment (22 ± 2 °C) with a 12-h light/12-h dark cycle (lights on at 0600 h) for at least 2 weeks before experiments. All procedures were approved by the animal care committee of Tokushima University.

2.2. Preparation and incubation of parotid tissue slices and acinar cells

Parotid glands from rats, wild-type and mutant mice lacking nNOS were transferred to ice-cold, oxygenated Krebs–Ringer Tris (KRT) solution, consisting of 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 16 mM Tris–HCl (pH 7.4), and 5 mM glucose. Tissue slices (0.4 mm thick) were prepared from the glands with a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK) and were equilibrated with KRT solution for 15 min at 37 °C with shaking, before the various experimental incubations. Incubations were performed with ~ 50 mg of tissue in a final volume of 10 ml of KRT in the presence or absence of SNI-2011 or other agents as indicated. In some experiments, rat parotid acinar cells were isolated by digestion of tissue with collagenase and hyaluronidase as described (Ishikawa et al., 1988).

2.3. Western blot analysis for nNOS, iNOS, and eNOS

The supernatant from isolated parotid acinar cells of wild-type mice and mutant mice lacking nNOS by the method of Resta et al. (1999) was dissolved and was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 7.5% linear polyacrylamide gel. After electrophoresis, the protein was transferred as described previously (Ishikawa et al., 2000). The blots were probed with anti-nNOS antibody, anti-iNOS antibody, or anti-eNOS antibody, diluted 1:1500, or the antibody preadsorbed with their respective excess immunizing synthetic peptide used as the antigen, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunodetection was performed according to the enhanced chemiluminescence method (Amersham).

2.4. Determination of NOS activity in parotid acinar cells

Isolated parotid acinar cells of rats were incubated for 30 min at 37 °C in RPMI 1640 medium (pH 7.4) that was supplemented with 10 μM 4,5-diaminofluorescein/diacetate (DAF-2/DA) and had been gassed with 95% O_2–5% CO_2. The cells were then washed and resuspended in HEPES-buffered Krebs–Ringer bicarbonate solution, consisting of 118.46 mM NaCl, 4.74 mM KCl, 1.18 mM KH_2PO_4, 1.00 mM $CaCl_2$, 1.18 mM $MgSO_4$, 24.88 mM $NaHCO_3$, and 5 mM HEPES–NaOH (pH 7.4), for measurement of NOS activity as described (Tritsaris et al., 2000). In brief, the cells were stirred gently in a cuvette maintained at 37 °C, with or without SNI-2011 and the other agents. DAF-2DA is taken

up by the cells and hydrolyzed by cellular esterases to form the membrane-impermeable compound DAF-2. DAF-2 reacts with NO in the presence of oxygen to form the fluorescent triazolofluorescein. The changes in the fluorescence were monitored with a fluorescence spectrometer (CF-4000; Hitachi, Tokyo, Japan). The excitation and emission wavelengths were 495 nm (5-nm bandwidth) and 515 nm (5-nm bandwidth), respectively.

2.5. Immunoblot analysis for muscarinic M_3 receptors

Basolateral membranes prepared from parotid glands of wild-type mice and mutant mice lacking nNOS by the method of Ishikawa et al. (1998) were dissolved in 200 μl of solubilizing buffer consisting of 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.04% 3-[(3-cholamidopropyl)dimethyl-ammonio]propanesulfonic acid (CHAPS), and then mixed with 10 μl of mercaptoethanol containing bromophenol blue. Aliquots (40 μg protein in each lane) were subjected to SDS-PAGE in 10% linear polyacrylamide gels. After electrophoresis, the separated proteins were transferred electrophoretically from the unstained gel to a nitrocellulose membrane (Hybond ECL; Amersham Bioscience, Little Chalfont, Buckinghamshire, UK) using a Trans Blot apparatus (Bio-Rad, Hercules, CA). After transfer, the nitrocellulose transfer membranes were quenched for 60 min in TBS-T (20 mM Tris–HCl buffer (pH 7.6), 137 mM NaCl, and 0.1% Tween-20) containing 5% low-fat dried milk. The membranes were then incubated with antibodies to muscarinic M_3 receptor protein (diluted 1:1000) in the same buffer for 18 h at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunodetection was performed according to the enhanced chemiluminescence (ECL) method (Amersham).

2.6. Assay of calmodulin kinase II and PKG activities in parotid tissue slices

After experimental incubations, parotid tissue slices were rapidly frozen at −80 °C. For measurement of calmodulin kinase II activity, the frozen tissues were homogenized in a solution containing 20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 2 mM EGTA, soybean trypsin inhibitor (20 μg/ml), aprotinin (10 μg/ml), leupeptin (5 μg/ml), 2 mM dithiothreitol, 25 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 350 × *g* for 5 min, and the resulting supernatant was diluted with a solution containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and bovine serum albumin (0.1 mg/ml) before assay of calmodulin kinase II activity with a specific kit (Gaithersberg, MD). For measurement of PKG activity, the frozen tissues were homogenized in a solution comprising 20 mM HEPES–NaOH (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 25 mM $MgCl_2$, 2 mM sodium orthovanadate, 140 mM NaCl, 1 mM dithiothreitol, and a mixture of protease inhibitors [1 mM Pefabloc, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)]. The homogenate was centrifuged for 15 min at 15,000 × *g*, and the resulting supernatant was assayed for PKG activity with a specific kit (Promega, Madison, WI, USA). In brief, 100 μl of the supernatant from the homogenate were added to 50 μl of assay mixture containing 20 mM Tris–HCl (pH 7.4), 200 μM ATP, 100 μM BPDEtide, 20 mM $MgCl_2$, 100 μM 1-methyl-3-isobutylxanthine, 1 μM 6-22 amide, and 0.5 μCi of [γ-^{32}P]ATP. After incubation for 10 min at 30 °C, the reaction was terminated by the addition of 140 μl of ice-cold 10% (w/v) trichloroacetic acid. The mixture was centrifuged for 5 min at 15,000 × *g* to separate precipitated proteins, and the resulting supernatant was spotted onto phosphocellulose filters. After removal of unreacted [γ-^{32}P]ATP, the filter-associated radioactivity was measured with a liquid scintillation spectrometer in order to determine the incorporation of ^{32}P into BPDEtide.

2.7. Other methods

The amylase activity of incubation medium was measured as described (Bernfeld, 1955) with amylose as the substrate, and was expressed as milligrams of maltose produced during incubation for 5 min at 20 °C.

2.8. Statistical analysis

Data are expressed as means ± S.E. and were tested for statistical significance with Student's *t*-test. A P value of < 0.05 was considered statistically significant.

2.9. Drugs and reagents

Anti-nNOS, anti-eNOS, and anti-iNOS and their respective immunizing synthetic peptides used as the antigen were obtained from BIOMOL Research Laboratories, (Plymouth Meeting, PA, USA). DAF-2/DA, hexahydro-sila-difenidol hydrochloride,*p*-fluoroanalog (*p*-F-HHSid), methoctramine, and 3,4,5-trimethoxy-benzoic 8-(diethylamino)octylester (TMB-8) were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Pilocarpine, carbachol, dantrolene, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Carboxy-PTIO), 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9), RPMI 1640 medium, and 1-[6-{[(17β)-3-methoxyestra-1,3,5-(10)-trien-17-yl]-amino}hexyl-1*H*-pyrrole-2,5-dione] (U-73122) were obtained from Sigma (St. Louis, MO, USA). (*N*-[2-{[(3-(4-chlorophenyl)-2-propenyl)metylamino]methl}phenyl]-*N*-(2-hydroxyethyl)-4-methoxy-benzenesulfonamine) (KN-93), N^G-nitro-L-arginine methylester hydrochloride (L-NAME), 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinoxaline-1-one (ODQ), and 3-morpholinosydnonimine (SIN-1) were from Funakoshi

(Tokyo, Japan). 2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*H*-tetraacetic acid acetoxymethyl ester (BAPTA-AM), ([9*R*, 10*S*,12*S*]-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3,2,1-ki]pyrrolo [3,4-1][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT5720), and 8*R*,9*S*,11*S*-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-trizadibenzo-(*a*,*g*)-cycloocta-(*c*,*d*,*e*)-trinden-1-one (KT5823) were from Calbiochem (Nottingham, UK).

3. Results

3.1. Effect of SNI-2011 on amylase secretion from rat parotid tissue slices

SNI-2011 induced amylase secretion from rat parotid tissue with a median effective concentration of 1.26 ± 0.1 μM, which is similar to that for pilocarpine (1.09 ± 0.1 μM), but is bigger than that for carbachol (Fig. 1). The comparison of the dose–response curve on amylase secretion for SNI-2011 with those for pilocarpine and carbachol showed that SNI-2011 acted at muscarinic M_3 receptors on rat parotid cells as a partial agonist and induced amylase secretion. The secretory response of the tissue to 10 μM SNI-2011 was rapid, being detectable at 1 min after exposure to this agent (Fig. 1). When parotid tissue slices were incubated with various concentrations of SNI-2011 in the presence of 1 μM carbachol, amylase was secreted from the tissues additively (data not shown). Amylase secretion induced by 10 μM SNI-2011 was completely inhibited by 100 μM *p*-F-HHSiD, a muscarinic M_3 receptor antagonist, but was unaffected by the same concentration of the muscarinic M_2 receptor antagonist, methoctramine, showing that this effect of SNI-2011 was mediated by muscarinic M_3 receptors (Table 1).

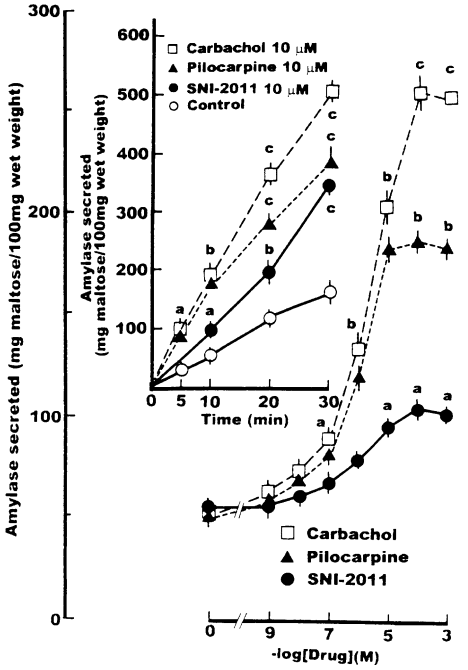

Fig. 1. Dose and time dependence of the effects of SNI-2011 and pilocarpine on amylase secretion from rat parotid tissue slices. Tissue slices were incubated for 10 min with the indicated concentrations of SNI-2011 or pilocarpine, or for the indicated times in the absence (control) or presence of 10 μM SNI-2011 or 10 μM pilocarpine (inset). The activity of amylase released into the incubation medium was then measured. Data are means ± S.E. of values from three or four experiments. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ versus corresponding control value.

Table 1
Effects of M_2 and M_3 receptor antagonists and U-73122 on SNI-2011-induced amylase secretion from rat parotid tissue slices

Agonist (μM)	Antagonist (μM)	Amylase activity (mg maltose/100 mg wet weight)
None	None	56.3 ± 1.2
	Methoctramine (100)	57.4 ± 0.7
	p-F-HHSiD (100)	54.9 ± 0.6
	U-73122 (5)	50.2 ± 1.8
	U-73122 (10)	42.6 ± 0.3
SNI-2011 (10)	None	111.1 ± 6.6[a]
	Methoctramine (100)	104.8 ± 5.7[a]
	p-F-HHSiD (100)	59.8 ± 2.9
	U-73122 (5)	61.1 ± 2.5
	U-73122 (10)	58.4 ± 2.3

Rat parotid tissue slices were preincubated for 10 min in the absence or presence of methoctramine, *p*-F-HHSiD or U-73122 before incubation for 10 min with or without SNI-2011 in the continued absence or presence of methoctramine, *p*-F-HHSiD or U-73122. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three or four experiments.

[a] $P<0.05$ versus value for the slices incubated alone.

3.2. Effect of $[Ca^{2+}]i$ on SNI-2011-induced amylase secretion from rat parotid tissue slices

Activation of M_3 receptors in rat parotid tissue slices induces the activation of phospholipase C (Sawaki et al., 1993). To determine whether the activation of phospholipase C contributes to the stimulatory effect of SNI-2011 on amylase secretion, we pretreated the slices with 5 and 10 μM U-73122, a selective inhibitor of phospholipase C, and then added 10 μM SNI-2011 in the continuous presence of this inhibitor. This inhibitor prevented the stimulatory effect of SNI-2011 on amylase secretion in a dose-dependent manner (Table 1).

The generation of inositol-1,4,5-trisphosphate (IP_3) by phospholipase C and the consequent mobilization of Ca^{2+} from intracellular stores might thus contribute to the effect of SNI-2011 on amylase secretion. This suggestion is supported by our observations that 15 μM TMB-8 (which inhibits Ca^{2+} release from IP_3-sensitive intracellular stores) and 15 μM dantrolene (which prevents the release of Ca^{2+} from IP_3-insensitive stores) each inhibited SNI-2011-induced amylase secretion (Table 2). In general, IP_3-gated channels release Ca^{2+} that, in turn, triggers Ca^{2+} release by

Table 2
Effect of $[Ca^{2+}]_i$ on SNI-2011-induced amylase secretion from rat parotid tissue slices in the absence or presence of extracellular $CaCl_2$

Experiment	Stimulant (μM)	Medium	Agent (μM)	Amylase activity (mg maltose/100 mg wet weight)
A	None	$CaCl_2$ free	None	35.2 ± 0.8
		EGTA	None	29.5 ± 0.6
	SNI-2011 (10)	$CaCl_2$ free	None	38.6 ± 2.2
		EGTA	None	23.8 ± 1.4
B	None	$CaCl_2$	None	45.2 ± 1.7
		$CaCl_2$	TMB-8 (15)	41.2 ± 3.2
		$CaCl_2$	Dantrolene (15)	32.6 ± 2.0
		$CaCl_2$	BAPTA-AM (100)	37.0 ± 2.0
	SNI-2011 (10)	$CaCl_2$	None	97.1 ± 7.3[a]
		$CaCl_2$	TMB-8 (15)	50.4 ± 1.2
		$CaCl_2$	Dantrolene (15)	38.9 ± 0.5
		$CaCl_2$	BAPTA-AM (100)	42.0 ± 1.6

Rat parotid tissue slices were incubated for 10 min with or without SNI-2011 in the absence (experiment A) or presence (experiment B) of 1 mM $CaCl_2$. Where indicated in experiment A, the tissue was also incubated in $CaCl_2$-free medium containing 1 mM EGTA. Where indicated in experiment B, rat parotid tissue slices were preincubated for 10 min in the absence or presence of TMB-8, dantrolene, or BAPTA-AM before incubation for 10 min with or without SNI-2011 in the continued absence or presence of TMB-8, dantrolene, or BAPTA-AM. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three or four experiments.

[a] $P < 0.05$ versus the value for the slices not exposed to any drug.

the pathway of Ca^{2+}-induced Ca^{2+} release, which is mediated by ryanodine receptors (Berridge, 1993). The influx of Ca^{2+} across the plasma membrane is generally required to sustain oscillations in $[Ca^{2+}]_i$ and to replace Ca^{2+} lost from the cell through the activity of Ca^{2+} extrusion mechanisms that operate at the plasma membrane (Foskett et al., 1991). Treatment of rat parotid tissue with 100 μM BAPTA-AM (Table 2), a cell-permeable Ca^{2+} chelator, or removal of extracellular $CaCl_2$ (Table 2) also inhibited SNI-2011-induced amylase secretion. Together, these results indicate that an increase in $[Ca^{2+}]_i$ caused by Ca^{2+} release from intracellular storage sites and Ca^{2+} entry from extracellular sites contribute to the stimulation by SNI-2011 of amylase secretion from parotid tissue.

3.3. Effects of KN-93, L-NAME, and ODQ on SNI-2011-induced amylase secretion from rat parotid tissue slices

To elucidate the mechanism by which SNI-2011-induced increase in $[Ca^{2+}]_i$ contributes to amylase secretion, we first examined the possible role of calmodulin kinase II. Pretreatment and treatment of rat parotid tissue slices with 10 μM KN-93, a selective inhibitor of calmodulin kinase II, completely blocked amylase secretion induced by 10 μM SNI-2011 (Table 3), suggesting that this enzyme participates in the Ca^{2+}-mediated secretory response to SNI-2011. To examine the possible role of NOS, we exposed the slices to 10 μM L-NAME, a selective inhibitor of nNOS. This agent prevented amylase secretion in response to 10 μM SNI-2011 (Table 3), suggesting that the activation of nNOS is important in SNI-2011-induced exocytosis and that the increase in $[Ca^{2+}]_i$ induced by SNI-2011 is sufficient to activate nNOS and thereby to generate NO. To assess the possible role of soluble guanylate cyclase, which is activated by NO, in SNI-2011-induced amylase secretion, we treated the slices with ODQ, a selective inhibitor of this enzyme. ODQ (10 μM) completely inhibited amylase secretion induced by SNI-2011 (Table 3), suggesting that cyclic GMP produced by soluble guanylate cyclase participates in amylase secretion triggered by SNI-2011. Collectively, these results indicate that Ca^{2+} signaling triggered by SNI-2011 results in the activation of calmodulin kinase II, nNOS, and soluble guanylate cyclase in rat parotid cells.

3.4. Effect of SNI-2011 on calmodulin kinase II in rat parotid tissue slices

To determine whether the increase in $[Ca^{2+}]_i$ induced by exposure of rat parotid tissue slices to SNI-2011 activates calmodulin kinase II and NOS, we measured the activities of these enzymes. Exposure of the tissue slices to 10 μM SNI-2011 for 3 min induced an approximately twofold increase in calmodulin kinase II activity (0.063 ± 0.021 and 0.121 ± 0.01 pmol/min/mg of protein for tissue slices incubated in the absence or presence of SNI-2011, respectively; means ± S.E. of values from four experiments, $P < 0.05$), consistent with our observation that KN-93 inhibited amylase secretion evoked by SNI-2011 (Table 3).

Table 3
Effects of KN-93, L-NIL, and ODQ on SNI-2011-induced amylase secretion from rat parotid tissue slices

Stimulant (μM)	Inhibitor (μM)	Amylase activity (mg maltose/100 mg wet weight)
None	None	52.4 ± 1.3
	KN-93 (1)	53.7 ± 1.2
	KN-93 (10)	52.2 ± 1.5
	L-NAME (1)	56.3 ± 1.0
	L-NAME (10)	54.9 ± 1.6
	ODQ (1)	50.2 ± 2.0
	ODQ (10)	55.2 ± 1.2
SNI-2011 (10)	None	102.9 ± 4.7[a]
	KN-93 (1)	82.3 ± 2.8
	KN-93 (10)	56.7 ± 1.7
	L-NAME (1)	82.9 ± 1.7
	L-NAME (10)	60.5 ± 0.6
	ODQ (1)	84.3 ± 2.5
	ODQ (10)	62.2 ± 2.6

Rat parotid tissue slices were preincubated for 10 min in the absence or presence of KN-93, L-NIL, or ODQ before incubation for 10 min with or without SNI-2011 in the continued absence or presence of KN-93, L-NIL, or ODQ. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three experiments.

[a] $P < 0.05$ versus the value for the slices not exposed to any drug.

3.5. Effect of SNI-2011 on NOS activities in rat parotid isolated cells

We investigated changes in the activity of endogenous NOS in isolated parotid acinar cells of rats after incubation with SNI-2011 in real time by using the fluorescent NO indicator DAF-2/DA. The cells were loaded with 10 μM DAF-2/DA and then challenged with 10 μM SNI-2011 in order to measure NO production. SNI-2011 induced a marked increase in DAF-2 fluorescence (Fig. 2), reflecting an increase in NO synthesis in the cells. The activation of NOS by SNI-2011 was prevented by preloading of the cells with 100 μM BAPTA-AM for 10 min (Fig. 2). A23187 (10 μM) also induced the production of NO in the cells; the effect of the ionophore was smaller than that observed with SNI-2011 (data not shown). These findings suggest that NO generation in rat parotid acinar cells is coupled to increase in $[Ca^{2+}]_i$. Exposure of the parotid acinar cells to the NO donor SIN-1 (1 μM) induced a large increase in DAF-2 fluorescence, demonstrating that NO reacted with DAF-2, resulting in the production of triazolofluorescein (data not shown). Together, these results indicated that nNOS is endogenously present in rat parotid acinar cells and is activated by the increase in $[Ca^{2+}]_i$ induced by SNI-2011.

3.6. Effects of carboxy-PTIO on SNI-2011-, SNAP and SIN-1-induced amylase secretion from rat parotid tissue slices

To determine whether NO production induced by SNI-2011 stimulates amylase secretion from rat parotid tissue slices, we examined the effects of an NO scavenger and an inhibitor of NOS on exocytosis in response to SNI-2011 and NO donors. An NO scavenger, carboxy-PTIO (300 μM), completely inhibited SNI-2011-induced amylase secretion (Table 4). The NO scavenger did not inhibit isoprenaline-induced amylase secretion (data not shown), showing that it does not affect exocytosis per se. On the contrary, SNAP and SIN-1, NO donors, markedly stimulated amylase secretion (Table 4). SNAP- and SIN-1-induced amylase secretion was also inhibited by carboxy-PTIO (Table 4). These results demonstrate that NO contributes to SNI-2011-induced amylase secretion from rat parotid tissue slices.

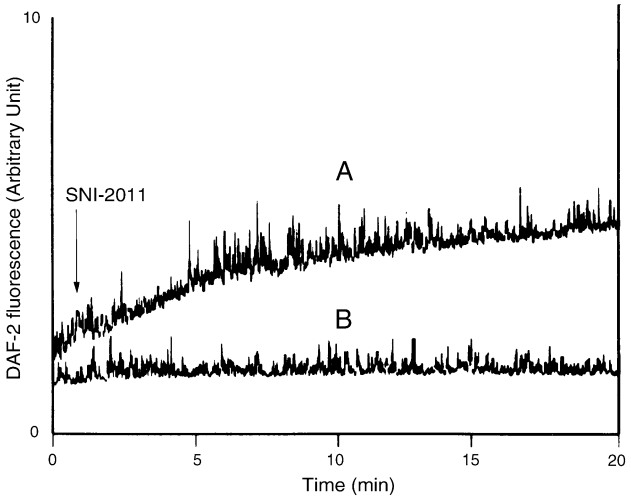

Fig. 2. Effect of SNI-2011 on NOS activity in rat parotid acinar cells. NOS activity was measured as the change in fluorescence intensity in isolated parotid acinar cells of rats that had been preincubated with 10 μM DAF-2/DA for 30 min (A and B). Cells were incubated in the absence (A) or presence (B) of 100 μM BAPTA-AM for 10 min and then further exposed to 10 μM SNI-2011 for the indicated times. All traces are representative of four or five separate experiments. SNI-2011 (A and B) was applied at the time indicated by the arrow.

Table 4
Effects of carboxy-PTIO on SNI-2011-, SNAP-, and SIN-1-induced amylase secretion from rat parotid tissue slices

Agonist (μM)	Inhibitors (μM)	Amylase activity (mg maltose/100 mg wet weight)
None	None	50.2 ± 0.7
	Carboxy-PTIO (100)	51.2 ± 1.0
	Carboxy-PTIO (300)	51.4 ± 1.4
SNI-2011 (10)	None	95.2 ± 3.1[a]
	Carboxy-PTIO (100)	75.2 ± 2.0
	Carboxy-PTIO (300)	47.2 ± 1.5
SNAP (0.1)	None	76.0 ± 5.2
SNAP (1)	None	142.8 ± 2.7[b]
SNAP (10)	None	183.2 ± 1.9[b]
SNAP (100)	None	226.5 ± 6.5[c]
SNAP (100)	Carboxy-PTIO (300)	51.8 ± 3.3
SIN-1 (10)	None	134.2 ± 4.3[b]
SIN-1 (100)	None	188.6 ± 5.5[b]
SIN-1 (100)	Carboxy-PTIO (300)	55.3 ± 3.8

Rat parotid tissue slices were preincubated for 10 min in the absence or presence of carboxy-PTIO before incubation for 10 min with or without SNI-2011, SNAP, or SIN-1 in the continued absence or presence of carboxy-PTIO. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three experiments.

[a] $P < 0.05$ versus value for the slices incubated alone.

[b] $P < 0.01$ versus value for the slices incubated alone.

[c] $P < 0.001$ versus value for the slices incubated alone.

3.7. Effects of KT5823 and KT5720 on SNI-2011- and SNAP-induced amylase secretion and of SNI-2011 on PKG activity in rat parotid tissue slices

To examine further the role of PKG activation by cyclic GMP (produced as a result of the activation of GC-S) in SNI-2011- and SNAP-induced amylase secretion, we investigated the effect of KT5823, a selective inhibitor of this enzyme. KT5823 inhibited does-dependently amylase secretion induced by 10 μM SNI-2011 as well as that induced by 1 μM SNAP (Table 5). On the other hand, KT5720, a selective inhibitor of cyclic AMP-dependent protein kinase (PKA), did not inhibit the exocytosis induced by these agents (Table 5). Incubation of rat parotid tissue slices with SNI-2011 (10 μM) for 3 min also induced an approximately twofold increase in PKG activity (6.76 ± 0.29 and

Table 5
Effects of KT5720 and KT5823 on SNI-2011- or SNAP-induced amylase secretion from rat parotid tissue slices

Stimulant (μM)	Inhibitor (μM)	Amylase activity (mg maltose/100 mg wet weight)
None	None	46.6 ± 3.2
	KT5720 (10)	45.5 ± 1.8
	KT5823 (5)	46.3 ± 1.5
	KT5823 (10)	48.4 ± 1.2
SNI-2011 (10)	None	89.9 ± 6.5[a]
	KT5720 (10)	95.1 ± 6.2[a]
	KT5823 (5)	60.5 ± 2.1
	KT5823 (10)	49.5 ± 2.7
SNAP (1)	None	148.5 ± 4.8[b]
	KT5720 (10)	122.0 ± 4.0[b]
	KT5823 (10)	41.0 ± 4.5

Rat parotid tissue slices were preincubated for 10 min in the absence or presence of KT5823 and KT5720 before incubation for 10 min with or without SNI-2011 and SNAP in the continued absence or presence of KT5823 or KT5720. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three experiments.

[a] $P < 0.05$ versus the value for the slices incubated alone.

[b] $P < 0.01$ versus the value for the slices incubated alone.

14.58 ± 0.81 fmol/mg of protein for tissue slices incubated in the absence or presence of SNI-2011, respectively; means ± S.E. of values from five experiments, $P < 0.05$). These observations thus show the implication of PKG in amylase secretion induced by SNI-2011.

3.8. Effect of ML-9 on SNI-2011-induced amylase secretion from rat parotid tissue slices

MLCK, a Ca^{2+}, calmodulin-dependent protein kinase, has been implicated in Ca^{2+}-dependent secretion (Mizuno et al., 2000). We investigated the effect of ML-9, a selective inhibitor of MLCK, on amylase secretion induced by SNI-2011. This inhibitor prevented amylase secretion in response to 10 μM SNI-2011 in a dose-dependent manner (Table 6), suggesting that MLCK plays an important role in stimulus–secretion coupling in rat parotid cells.

3.9. Effect of SNI-2011 on amylase secretion from parotid tissue slices of wild-type and mutant mice lacking nNOS

We used mice with the deletion in nNOS gene to confirm the role of nNOS in the induction by SNI-2011 of amylase secretion from parotid tissue slices. The deficiency of nNOS expression was verified by Western blot analysis for nNOS (Fig. 3), and lack of NOS activity was determined in parotid acinar cells isolated from nNOS knockout mice by using the fluorescent NO indicator DAF-2/DA (data not shown). The expression of M_3 muscarinic receptor was not different in nNOS knockout mice compared to wild-type mice (Fig. 3). The total content of amylase in parotid tissues from mutant mice lacking nNOS here was 15% decrease in comparison with that of wild-type mice (551.8 ± 19.7 and 479.8 ± 13.8

Table 6
Effect of ML-9 on SNI-2011-induced amylase secretion from rat parotid tissue slices

Agonist (μM)	ML-9 (μM)	Amylase activity (mg maltose/100 mg wet weight)
None	0	51.6 ± 1.5
	10	50.7 ± 1.7
	20	41.2 ± 1.1
SNI-2011 (10)	0	113.1 ± 6.0[a]
	10	80.5 ± 1.5
	20	47.2 ± 1.7

Rat parotid tissue slices were preincubated for 10 min in the absence or presence of ML-9 before incubation for 10 min with or without SNI-2011 in the continued absence or presence of ML-9. The incubation medium was then assayed for amylase activity. Data are means ± S.E. of values from three experiments.

[a] $P < 0.05$ versus value for the slices incubated without ML-9 and SNI-2011.

mg maltose/100 mg wet weight in parotid tissues from wild-type and mutant mice lacking nNOS, respectively). As shown in Fig. 4(B), the tissue from nNOS knockout mice did not show the secretory response to 1 and 10 μM SNI-2011. However, isoproterenol (1 μM) caused rapid and significant amylase secretion from nNOS knockout mouse parotid tissues (amylase secreted from parotid tissues incubated for 10 min with or without 1 μM isoproterenol in nNOS knockout mouse: 9.7 ± 0.6 and 58.2 ± 5.3 mg maltose/5 min/100 mg wet weight, respectively), showing that parotid acinar cells in the knockout mice were functional to β-adrenergic stimulation. On the other hand, the tissue from wild-type mice showed rapid and strong secretory response to 1 and 10 μM SNI-2011 as observed in rat parotid tissues (Fig. 4(A)). The SNI-2011-induced amylase secretion from wild-type mice parotid tissues was completely inhibited by 100 μM *p*-F-HHSid, 10 μM KN-93, 10 μM L-NAME, and 10 μM ODQ as in the case of rat parotid tissues (data not

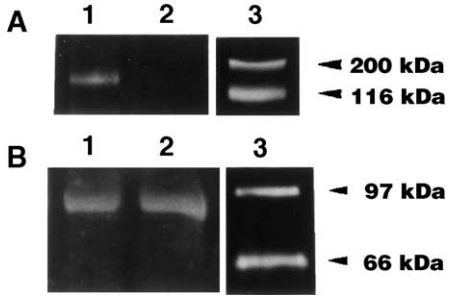

Fig. 3. Western blots for nNOS and muscarinic M_3 receptors in mouse parotid acinar cells. The supernatant from homogenate (A) and basolateral membranes (B) of isolated parotid acinar cells of wild-type mice (1) and nNOS knockout mice (2) was subjected to SDS-PAGE and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The blots were proved with anti-nNOS antibody (1:1,500 dilution) (A) and anti-muscarinic M_3 receptor antibody (1:1,500 dilution) (B). Immune complex was detected by enhanced chemiluminescence. Profile of electro-chemiluminescent protein molecular weight markers are shown in lane 3.

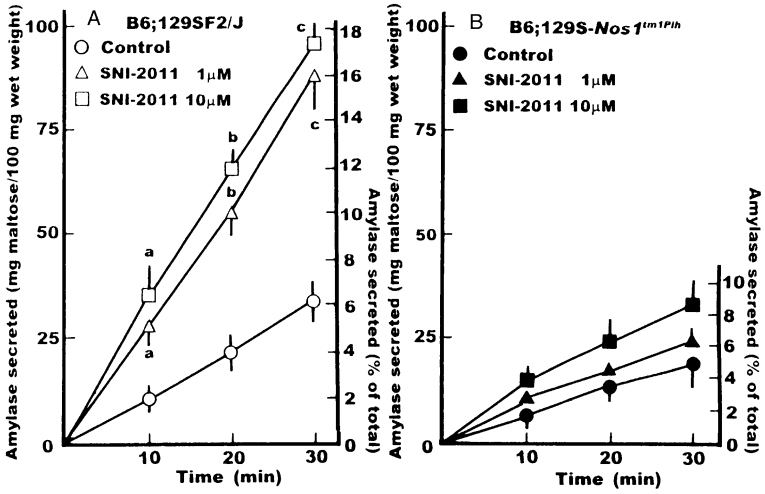

Fig. 4. Effect of SNI-2011 on amylase secretion from parotid tissue slices of wild-type mice and mutant mice lacking nNOS. Tissue slices prepared from wild-type mice (B6; 129SF2/J101045) (A) and mutant mice lacking nNOS (B6; 129S-NOS 1^{tm1plh}) (B) were incubated with or without 1 μM SNI-2011 for the indicated times. The activity of amylase released into the incubation medium was then measured. The left longitudinal axis was expressed as milligrams of maltose produced during incubation. The right longitudinal axis was expressed as the amylase secreted (% of the total content of amylase in parotid tissues). Data are means ± S.E. of values from four experiments. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ versus corresponding control value.

shown). These results indicated the importance of the activation of nNOS in Ca^{2+} signaling triggered by SNI-2011 in parotid acinar cells.

4. Discussion

We have shown that SNI-2011 acts at muscarinic M_3 receptors on rat parotid acinar cells and induces amylase secretion in a dose-dependent manner (Table 1; Fig. 1). SNI-2011-induced amylase secretion was blocked by the phospholipase C inhibitor, U73122 (Table 2). The activation by SNI-2011 of M_3 receptors on parotid acinar cells induces the mobilization of Ca^{2+} from intracellular stores as a result of the generation of IP_3 by phospholipase C (Ishikawa et al., 2000). We have now shown that exposure of rat parotid tissue to TMB-8, dantrolene, or BAPTA-AM completely inhibited SNI-2011-induced amylase secretion, suggesting that this effect of SNI-2011 depends on increase in $[Ca^{2+}]_i$ caused by Ca^{2+} release from intracellular storage sites and Ca^{2+} entry from extracellular sites in rat parotid cells (Table 2). Calmodulin is an important effector of Ca^{2+} signaling in many mammalian cell types, and the Ca^{2+}–calmodulin complex activates various protein kinases, including the multifunctional enzyme calmodulin kinase II. The selective calmodulin kinase II inhibitor, KN-93, completely blocked amylase secretion induced by SNI-2011, suggesting that the activation of this enzyme contributes to the stimulatory action of SNI-2011 on the exocytosis (Table 3). Calmodulin kinase II is thought to be required both for granule mobilization by acetylcholine and for the maintenance of secretory capacity under basal conditions in mouse pancreatic β cells (Gromada et al., 1999). The MLCK inhibitor ML-9 also completely inhibited SNI-2011-induced amylase secretion, implicating this calmodulin-dependent kinase in this process (Table 6). We previously showed that, in the presence of extracellular Ca^{2+}, SNI-2011 induced long-lasting increase in $[Ca^{2+}]_i$ in rat parotid acinar cells (Ishikawa et al., 2000). The increase in $[Ca^{2+}]_i$ evoked by physiological concentrations of cholecystokinin in pancreatic acinar cells was previously shown to be gradually reduced in magnitude by wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, or by the removal of Ca^{2+} from the extracellular space (Bragado et al., 1997). These observations suggest that MLCK regulates capacitative Ca^{2+} entry after depletion of intracellular Ca^{2+} stores, and that MLCK contributes to Ca^{2+}-stimulated amylase secretion. In addition, MLC phosphorylation by MLCK is thought to modulate the movement of secretory granules, resulting in enhanced insulin secretion in pancreatic β cells (Iida et al., 1997). Treatment of parotid tissue with L-NAME, a selective inhibitor of nNOS, prevented amylase secretion in response to SNI-2011, suggesting that SNI-2011-induced activation of nNOS contributes to the stimulatory action of this agonist on exocytosis (Table 3). This suggestion is supported by the results described as follows. It was also revealed by using isolated parotid acinar cells of rats loaded with DAF-2/DA that stimulation with SNI-2011 of the cells induced a fast increase in DAF fluorescence corresponding to an increase in the NO synthesis (Fig. 3). Recently, it was reported that carbachol activated nitric oxide synthase through a predominant muscarinic M_3 and a minor muscarinic M_1 receptors in rat parotid gland (Rosignoli and Perez Leiros, 2002). ODQ, a selective inhibitor of soluble guanyl cyclase, also markedly inhibited SNI-2011-induced amylase secretion (Table 3), suggesting that NO produced as a result of the SNI-2011- and SNAP-induced increase in nNOS activity activates soluble guanyl cyclase and that the consequent accumulation

of cyclic GMP contributes to amylase secretion. This suggestion is supported by our observation that the NO donor SNAP induced amylase secretion and that the NO scavenger carboxy-PTIO conversely inhibited SNI-2011-induced amylase secretion (Table 4). We also showed that SNI-2011 induced the activation of PKG, and that KT5823, a selective inhibitor of PKG, abolished the stimulatory effects of SNI-2011 and SNAP on amylase secretion; KT5720, a selective inhibitor of PKA, had no effect on SNI-2011-induced exocytosis (Table 5). These data show that PKG is implicated in the mechanism by which SNI-2011 induces amylase secretion.

With regard to possible substrates for calmodulin kinase II that participate in the stimulation of amylase secretion by SNI-2011, phosphorylation of synapsin I by this enzyme in neurons results in the dissociation of synaptic vesicles from the cytoskeleton and thereby facilitates vesicle translocation and fusion with the plasma membrane (Linas et al., 1991). Organized region in pancreatic β cells also requires Ca^{2+}–calmodulin-dependent phosphorylation of MLC (Niwa et al., 1998). Calmodulin kinase II associates with secretory granules in insulinoma cells (Möhling et al., 1997), and the substrates for this enzyme in pancreatic β cells include a subunit of tubulin (Colca et al., 1983), microtubule-associated protein 2 (Krueger et al., 1997), and MLC (Niki et al., 1993). These observations suggest that this kinase regulates various aspects of the interaction between secretory granules and the cytoskeleton. In regard of possible substrates for PKG in the exocytosis, the activation of this enzyme as a result of the accumulation of cyclic GMP has been shown to trigger the phosphorylation of a protein possibly involved in the exocytosis of synaptic vesicles (Yawo, 1999). The septin family of GTPases is associated with exocytosis and also includes a substrate for PKG (Xue et al., 2000). Furthermore, PKG may regulate MLCK.

We also compared the difference of secretory response of parotid tissues in wild-type and nNOS mutant mice to SNI-2011. The deficiency of nNOS expression in nNOS mutant mice used in this study was verified by Southern and Western blots, NOS immunostaining, NADPH-diaphorase staining, and NOS activity (Huang et al., 1993; Huang et al., 1994; Irikura et al., 1995). SNI-2011-induced amylase secretion in parotid tissues from wild-type mice was clearly observed, but was not in the tissues from mutant mice lacking nNOS, indicating strongly the importance of NO-dependent mechanisms in amylase secretion in parotid tissues induced by SNI-2011 (Fig. 4).

In summary, our data implicate calmodulin kinase II and NOS-PKG signaling pathway in the induction by SNI-2011 of amylase secretion from rat parotid acinar cells.

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