Persistent increase in the amount of aquaporin-5 in the apical plasma membrane of rat parotid acinar cells induced by a muscarinic agonist SNI-2011

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Abstract SNI-2011 induces the long-lasting increase in the amount of aquaporin-5 (AQP5) in apical plasma membranes (APMs) of rat parotid acini in a concentration-dependent manner. This induction was inhibited by *p*-F-HHSiD, U73122, TMB-8, or dantrolene but not by bisindolmaleimide or H-7, indicating that SNI-2011 acting at M_3 muscarinic receptors induced translocation of AQP5 via $[Ca^{2+}]_i$ elevation but not via the activation of protein kinase C. In contrast, acetylcholine induced a transient translocation of AQP5 to APMs. SNI-2011 induces long-lasting oscillations of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Thus, SNI-2011 induces a long-lasting translocation of AQP5 to APMs coupled with persistent $[Ca^{2+}]_i$ oscillations.

Key words: Apical plasma membrane; Aquaporin-5; Calcium oscillation; M_3 muscarinic receptor; Parotid acinar cell; SNI-2011

1. Introduction

Aquaporin proteins (AQPs), which form water channels, have been cloned from a variety of mammalian tissues [1–4]. In several non-excitable cell types, the function of AQPs is regulated by the interaction of hormones or neurotransmitters with their specific receptors. AQP1 in rat bile duct cells and AQP2 in rat renal collecting duct cells were transferred from the intracellular membrane (ICM) to the plasma membrane in response to secretin [5] and vasopressin [6,7], respectively, with a simultaneous increase in fluid transport. While we demonstrated that AQP5, which was cloned from rat submandibular glands [8], was transferred from the ICM to the apical plasma membrane (APM) in response to acetylcholine (ACh) or epinephrine via the activation of M_3 muscarinic receptors (M_3AChRs) or α_1-adrenergic receptors on the basolateral membrane (BLM), respectively [9,10]. Moreover, the maintenance of AQP5 in the APM was transient in parotid glands stimulated by ACh or epinephrine with a maximum at 15 s or 1 min, respectively.

The condition of xerostomia is characterized by oral dryness and difficulty in performing oral functions, and an impairment of fluid secretion in salivary glands is the etiology of this condition. In order to cure of xerostomia, we have looked for medical treatments which maintain persistently AQP5 in the APM of salivary glands. We have now found that treatment of the tissues with (±)-*cis*-2-methylspilo(1,3-oxathiolane-5,3′) quinuclidine (SNI-2011), which is a structurally rigid analog of ACh [11], induces a persistent increase in the amount of AQP5 in the APM via long-lasting $[Ca^{2+}]_i$ oscillations in the comparison with those of ACh in rat parotid cells.

2. Materials and methods

2.1. Preparation and incubation of rat parotid tissues

Parotid gland slices (300 mg wet weight, 0.4 mm thick) were prepared from male Wistar rats with a McIllwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK) and equilibrated with Krebs–Ringer–Tris (KRT) solution (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), 5 mM glucose) that had been gassed with O_2 at 37°C [12–14]. The slices were then incubated in 10 ml of fresh KRT solution in the absence or presence of SNI-2011 or other agents.

2.2. Preparation of APM, BLM and ICM fractions from rat parotid tissues

After the incubation, tissue slices were homogenized in 20 volumes of ice-cold 5 mM HEPES–Tris (pH 7.5) containing 50 mM mannitol and 0.25 mM $MgCl_2$ (buffer 1) with a glass homogenizer and a Teflon pestle. The homogenate was filtered through a single layer of nylon bolting cloth (150 mesh), and the filtrate was used to prepare APM, BLM and ICM fractions by the method of Longbottom and Van Heyningen [15], with a slight modification [9]. Briefly, the supernatant obtained at $35\,000\times g$ for 30 min at 4°C was centrifuged at $200\,000\times g$ for 1 h to obtain ICMs. The pellet from $35\,000\times g$ for 30 min at 4°C was suspended in buffer 1, followed by the addition of 1 M $MgCl_2$ to a final concentration of 10 mM and then left on ice for 30 min. The suspension was centrifuged at $3000\times g$ for 15 min and the resultant precipitate was used as BLMs. The resultant supernatant was again centrifuged at $35\,000\times g$ for 30 min to precipitate APMs. The activities of γ-glutamyltranspeptidase (γ-GT), as a marker of the APM, and of K^+-stimulated *p*-nitrophenyl-phosphatase (K*p*NPPase), as a marker of the BLM, were measured.

2.3. Measurement of $[Ca^{2+}]_i$ in parotid acinar cells

Parotid acinar cells, which were prepared by collagenase/hyaluronidase digestion as previously described [16], were subjected to measurement of $[Ca^{2+}]_i$ according to fluorescence study with fura-2/AM. The cells were gently stirred in a cuvette maintained at 37°C during the assay. Changes in fura-2/AM fluorescence were monitored with a fluorescence spectrometer (F-4000; Hitachi, Tokyo, Japan). The

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Abbreviations: SNI-2011, (±)-*cis*-2-methylspilo(1,3-oxathiolane-5,3′) quinuclidine; *p*-F-HHSiD, hexahydro-sila-difenidol; TMB-8, 4,5-trimethoxy-benzoic 8-(diethylamino) octylester; U73122, 1-6-[6-[[(17β)-3-methoxyestra-1,3,5-(10)-trien-17-yl]-amino]hexyl-1*H*-10ymole-2,5-dione]; GF 109203X, bisindolmaleimide; PMA, phorbol 12-myristate 13-acetate; PKA, protein kinase A; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate

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$[Ca^{2+}]_i$ was calculated from the ratio (340/380 nm) of fluorescence intensities after subtraction of autofluorescence, as described previously [16].

2.4. Immunoblot analysis

Rabbit polyclonal antibodies to AQP5 were generated in response to a synthetic peptide (KGTYEPEEDWEDHREERKKTI) corresponding to the deduced COOH-terminal amino acid sequence of AQP5 [8]. APM, BLM and ICM fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12.5% gels, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The blots were probed with anti-AQP5 antibody (1:1500 dilution) and anti-Na^+/K^+-ATPase antibody (1:50 dilution) (Chemicon, Temecula, CA, USA), or with the antibody that had been preadsorbed with excess synthetic peptide antigen. Immune complexes were detected by enhanced chemiluminescence.

2.5. Measurement of inositol 1,4,5-trisphosphate (IP_3) contents

The amount of IP_3 in rat parotid tissues was measured with the [^{3}H]IP_3 radioreceptor assay system (Du Pont NEN, Boston, MA, USA). After incubation of tissue slices as described above, IP_3 was extracted from the slices (100 mg wet weight) by the method of Zhang and Melvin [17]. Recovery from the entire extraction procedure was $>90\%$.

2.6. Statistical analysis

Data are presented as means ± S.E.M. and were analyzed for statistical significance with Student's *t*-test or analysis of variance. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Isolation of rat parotid membranes

To ascertain the contamination in APM, ICM and BLM fractions, γ-GT was used as a marker of the APM and K*p*NPPase and Na^+/K^+-ATPase were as markers of the BLM. The γ-GT activities in APMs, ICMs and BLMs were 375.0 ± 5.6, 18.3 ± 0.9 and 28.5 ± 2.7 nmol/min/mg protein, respectively. The K*p*NPPase activities were 36.9 ± 3.6, 11.1 ± 1.2 and 61.8 ± 4.3 nmol/min/mg protein in APMs, ICMs and BLMs, respectively. These fractions were then subjected to SDS–PAGE and transferred to nitrocellulose membranes. The blots were then probed with specific anti-Na^+/K^+-ATPase antibody. This antibody recognized a marked band with a molecular mass of 45 kDa in BLMs, a weak band in ICMs and no band in APMs (Fig. 1B). The densities of bands were not changed even in APMs, ICMs and BLMs prepared from tissues incubated with 10 μM SNI-2011. Thus, little of contaminating membranes in APMs and BLMs was determined by the two independent procedures described above and SNI-2011 did not affect the membrane fractionation.

The immunoblot analysis with the antibody to AQP5 recognized a clear solitary band with a mobility corresponding to the predicted molecular mass of AQP5 (27 kDa) in APMs and ICMs but not in BLMs (Fig. 1A). The treatment of tissues with 10 μM SNI-2011 increased the amount of AQP5 in APMs (Fig. 1A).

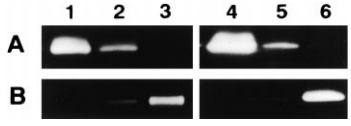

Fig. 1. Distributions of AQP5 and Na^+/K^+-ATPase in the APM, ICM and BLM of rat parotid glands. Parotid tissue slices were incubated with (lanes 4–6) or without (lanes 1–3) 10^{-5} M SNI-2011. APMs, ICMs and BLMs (20 μg of protein for AQP5 and 40 μg of protein for Na^+/K^+-ATPase) from rat parotid tissues were subjected to immunoblot analysis with antibodies to AQP5 (A) and Na^+/K^+-ATPase (B).

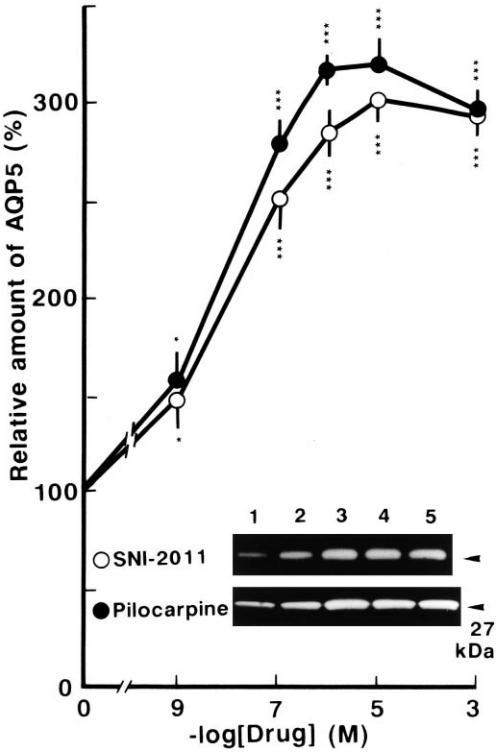

Fig. 2. Effects of various concentrations of SNI-2011 and pilocarpine on the amount of AQP5 in the APM. The APMs (5 μg of protein) from parotid tissues incubated for 10 and 3 min with the indicated concentrations of SNI-2011 or pilocarpine, respectively, were subjected to immunoblot analysis with antibody to AQP5. The blots were subjected to densitometric analysis, and the amount of AQP5 in the APMs was expressed as a percentage of the value for the tissues incubated in the absence of the agents. Data are means ± S.E.M. of 3–5 independent experiments. $*P<0.05$, $***P<0.001$ versus the value for control cells. The lower inset shows the results of a typical immunoblot of APMs of the tissues incubated with SNI-2011 or pilocarpine at concentrations of 0, 10^{-9}, 10^{-7}, 10^{-5}, or 10^{-3} M (lanes 1–5, respectively). The arrow is the position of 27 kDa.

3.2. Effect of SNI-2011 on the amount of AQP5 in the APM

The SNI-2011-induced increase in the amount of AQP5 in the APM was concentration-dependent (Fig. 2); it was significant at 1 nM and maximal (301.8 ± 9.2% of control) at 10 μM, with a median effective concentration (EC_{50}) value of 14.2 ± 2.3 nM, comparable to that for pilocarpine (9.0 ± 0.9 nM).

The SNI-2011-induced increase in the amount of AQP5 in the APM was detectable at 1 min, maximal at 10 min, and still significantly higher than that of control tissues after 30 min (Fig. 3). The effect of 10 μM pilocarpine was maximal at 3 min and still apparent after 30 min. In contrast, the effect of 10 μM ACh on the amount of AQP5 was maximal at 15 s and was no longer apparent after 10 min. Incubation of the tissue slices with 10 μM SNI-2011 for 10 min induced a significant increase in the amount of AQP5 in the APM and a corresponding decrease in its amount in the ICM (Fig. 4). In the BLM, the antibody to AQP5 did not recognize the expression of AQP5 (Fig. 1A). These results suggest that the accumulation

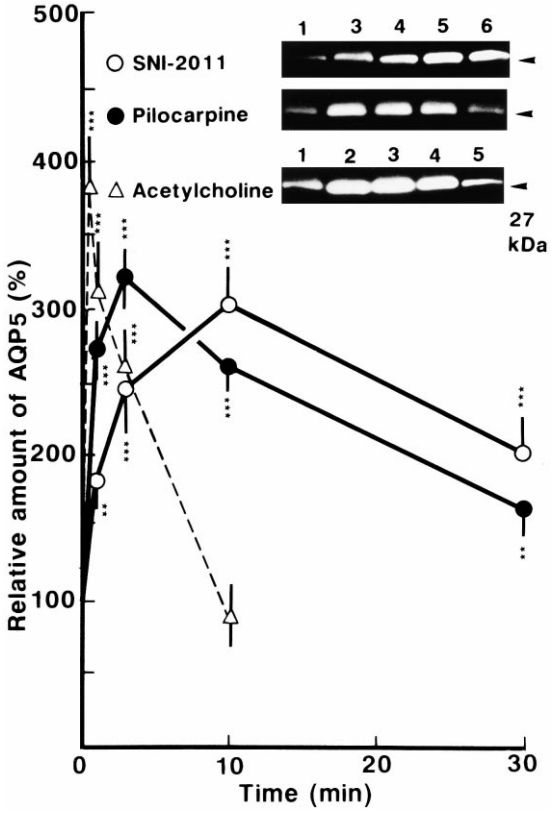

Fig. 3. Time courses for the effects of SNI-2011, pilocarpine, and ACh on the amount of AQP5 in the APM. The APMs (5 μg of protein) from parotid tissues incubated with SNI-2011 (10 μM), pilocarpine (10 μM), or ACh (10 μM) plus eserine (an inhibitor of acetylcholine esterase) (10 μM) for 0, 0.25, 1, 3, 10, or 30 min (corresponding to lanes 1–6, respectively, in the inset) were subjected to immunoblot analysis with antibody to AQP5. Data were quantitated by densitometry and are means ± S.E.M. of 3–5 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the value for control cells.

of AQP5 in the APM induced by SNI-2011 results from the fusion of AQP5-containing ICM. The protein concentrations in APM, BLM and ICM fractions from the parotid tissue homogenate (23.3 ± 1.5 mg protein) were 0.089 ± 0.008, 1.54 ± 0.10 and 2.23 ± 0.04 mg protein, respectively, showing

Table 1
Effects of various agents on the amount of AQP5 in the APM

Agent (μM)	Relative amount of AQP5 (%)
None	100
SNI-2011 (10)	301.8 ± 9.2***
SNI-2011 (10)+atropine (100)	100.4 ± 5.1
SNI-2011 (10)+*p*-F-HHSiD (10)	141.1 ± 14.3
SNI-2011 (10)+U73122 (10)	95.6 ± 2.4
SNI-2011 (10)+TMB-8 (30)	94.9 ± 1.6
SNI-2011 (10)+dantrolene (30)	140.7 ± 9.6
SNI-2011 (10)+H-7 (300)	254.4 ± 4.8***
SNI-2011 (10)+GF 109203X (10)	305.0 ± 14.4***
PMA (1)	90.7 ± 3.9

The APMs (5 μg of protein) from parotid tissues incubated for 10 min in the absence or presence of the indicated agents were subjected to immunoblot analysis with antibody to AQP5. The amount of AQP5 in the APM was quantitated by densitometry and expressed as described in the legend for Fig. 2. Data are means ± S.E.M. of 3–6 independent experiments. Significantly different from the control value, $^{***}P < 0.001$.

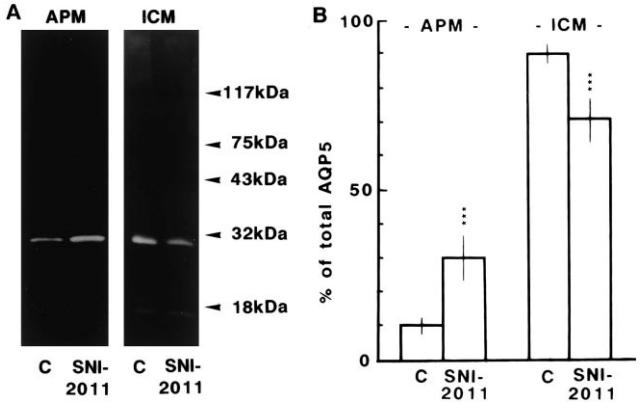

Fig. 4. Effect of SNI-2011 on the amount of AQP5 in the APM and the ICM. A: APMs (5 μg of protein) and ICMs (40 μg of protein) from parotid tissues incubated in the absence (C) or presence (SNI-2011) of 10 μM SNI-2011 for 10 min were subjected to immunoblot analysis with antibodies to AQP5. B: Immunoblots shown in A were subjected to densitometric analysis, and the amount of AQP5 in each fraction was expressed as % of total amount (the amount of AQP5 in APMs plus that in ICMs) of AQP5. Data are means ± S.E.M. of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus the value for control cells.

that the yields of APM, BLM and ICM fractions were 0.4, 7 and 10%, respectively. Treatment of the tissues with SNI-2011 or pilocarpine did not affect the yield of each fraction, nor did it affect immunoblot analysis per se.

Contrary to the finding that ACh or epinephrine induces the transient increase in the amount of AQP5 in the APM [9,10], these findings show that SNI-2011 or pilocarpine induces the long-lasting maintenance of AQP5 in the APM of rat parotid glands.

3.3. *Effects of atropine or hexahydro-sila-difenidol (p-F-HHSiD) on the SNI-2011-induced increase in the amount of AQP5 in the APM*

As shown in Table 1, the SNI-2011-induced increase in the abundance of AQP5 in the APM was prevented by 100 μM atropine and 10 μM *p*-F-HHSiD. This result is consistent with the observations that rat parotid glands express both M_2 and M_3 AChRs [18], and that M_3 AChRs constitute 90% of the total number of precipitable AChR in the tissues [19]. The interaction of SNI-2011 with M_3 AChRs induces the long-lasting maintenance of AQP5 in the APM of rat parotid glands.

3.4. *Effect of $[Ca^{2+}]_i$ on the amount of AQP5 in the APM*

Stimulation of M_3 AChRs on salivary glands with their respective agonists induces the generation of IP_3 and diacylglycerol; the former induces the release of Ca^{2+} from intracellular stores and the latter activates protein kinase C (PKC) [20,21]. Treatment of the tissues with either 300 μM H-7 or 10 μM bisindolmaleimide (GF 109203X), an inhibitor of PKC, had no marked effect on the amount of AQP5 in the APM in the tissues stimulated by SNI-2011 (Table 1). Conversely, 1 μM phorbol 12-myristate 13-acetate (PMA), an activator of PKC, did not induce an increase in the amount of AQP5 in the APM (Table 1). These results suggest that the SNI-2011-induced increase in the amount of AQP5 in the APM is not mediated by the activation of PKC. Incubation of the tissue slices with 30 μM 4,5-trimethoxy-benzoic 8-(di-

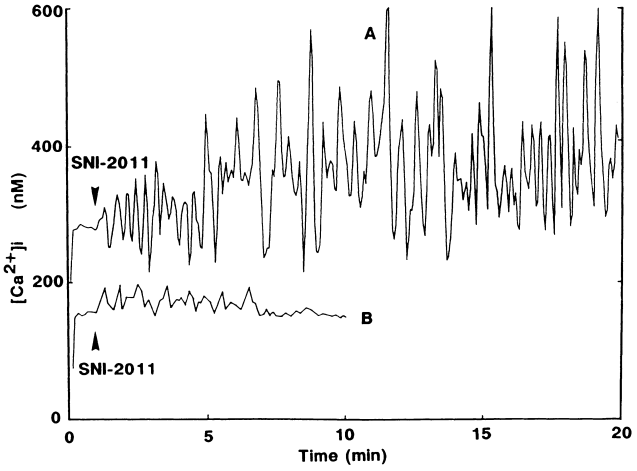

Fig. 5. Effect of SNI-2011 on $[Ca^{2+}]_i$ in rat parotid acinar cells. Isolated acinar cells were loaded with fura-2/AM and then exposed to 10 μM SNI-2011 in the presence (A) or absence (B) of 1 mM $CaCl_2$. The $[Ca^{2+}]_i$ was calculated from fluorescence measurements as described in Section 2. SNI-2011 was added at the time indicated by the arrow.

ethylamino) octylester (TMB-8), an inhibitor of calcium release from intracellular stores, prevented the increase in the amount of AQP5 in the APM induced by SNI-2011 (Table 1), indicating that the elevation of $[Ca^{2+}]_i$ associated with the activation of M_3 AChRs by SNI-2011 contributes to the effect on the amount of AQP5. 1-6-[6-[[(17β)-3-Methoxyestra-1,3,5-(10)-trien-17-yl]-amino]hexyl-1*H*-10ymole-2,5-dione] (U73122) completely inhibited the SNI-2011-induced increase in the amount of AQP5 in the APM at a concentration of 10 μM that blocked phosphoinositide hydrolysis by phospholipase C (PLC) and the generation of IP_3 (Table 1). In general, IP_3-gated channels might release Ca^{2+} which would in turn induce Ca^{2+} release from the Ca^{2+}-induced Ca^{2+} release pathway through ryanodine receptors (RyRs) [22]. Treatment of rat parotid tissue slices with 30 μM dantrolene, which prevents the release of Ca^{2+} from IP_3-insensitive stores, inhibited the SNI-2011-induced increase in the amount of AQP5 in the APM by 80% (Table 1).

Taken together, these results indicate that the SNI-2011-induced increase in the amount of AQP5 in the APM is mediated by an increase in $[Ca^{2+}]_i$ that results from Ca^{2+} release from intracellular stores through two distinct classes of receptors, IP_3 receptors and RyRs, but not by the activation of PKC.

3.5. Effect of SNI-2011 on the generation of IP_3

Incubation of the tissues with SNI-2011 caused an increase in the intracellular amount of IP_3; this effect was maximal at 10 min (3.20 ± 0.07 and 9.16 ± 1.42 pmol/mg protein in the control and SNI-2011-treated tissues, respectively) and remained apparent at 30 min (3.48 ± 0.34 and 5.78 ± 0.92 pmol/mg protein in the control and SNI-2011-treated tissues, respectively). The tissues stimulated by ACh generated IP_3 with a maximum at 15 s (3.89 ± 0.02 and 16.9 ± 2.2 pmol/mg protein in the control and ACh-treated tissues, respectively). These data suggest that the increase in the generation of IP_3 by SNI-2011 triggers the increase in the amount of AQP5 in the APM.

3.6. Effect of SNI-2011 on the oscillation of $[Ca^{2+}]_i$ in isolated parotid cells

To determine whether SNI-2011 mobilizes Ca^{2+} from intracellular pools, we loaded isolated parotid cells with fura-2/AM and then exposed them to 10 μM SNI-2011 in the absence or presence of 1 mM $CaCl_2$. SNI-2011 induced the generation of marked oscillations of $[Ca^{2+}]_i$ that persisted for longer than 30 min in the presence of extracellular $CaCl_2$ (Fig. 5). Although SNI-2011 also induced $[Ca^{2+}]_i$ oscillations in cells incubated in Ca^{2+}-free medium, both the $[Ca^{2+}]_i$ and the amplitude of the oscillations were greatly reduced in comparison with those apparent in cells incubated in Ca^{2+}-containing medium; the oscillations were also no longer detected after 6 min of the incubation. These results demonstrate that SNI-2011 mobilizes Ca^{2+} from intracellular stores and leads to the induction of a persistent increase in the amount of AQP5 in the APM.

4. Discussion

With the use of an AQP5-specific antiserum, we have now shown that SNI-2011 acting at M_3 AChRs induces a long-lasting increase in the amount of AQP5 in the APM in rat parotid glands (Fig. 3, Table 1). The long-lasting increase in the abundance of AQP5 in the APM induced by SNI-2011 was accompanied by a corresponding decrease in the amount of AQP5 associated with the ICM (Fig. 4). Intravenous administration of SNI-2011 induces long-lasting salivary secretion in rats and dogs [23]. Although the site of action of SNI-2011 on the long-lasting salivation had not been clear, we demonstrated that the increase in the amount of AQP5 in the APM of rat parotid glands participated in SNI-2011-induced salivation.

Recently, attention has been focused on the role of AQPs in each membrane fraction [1–3,9,10,24–26]. Several papers reported that the amount of AQP5 in the APM was regulated by neurotransmitters in salivary glands [9,10,26]. The exposure of rat parotid tissues to ACh or epinephrine induced increases in the amount of AQP5 in the APM [9,10]. Conversely, the treatment of parotid gland cells with β-adrenergic agonists resulted in the dispersal of AQP5 immunoreactivity as clusters of dots in submembranous cytoplasmic areas [26]. The ACh- or epinephrine-induced increase in the amount of AQP5 was no longer apparent after 10 min. In contrast, the effects of SNI-2011 and pilocarpine on the amount of AQP5 in the APM were maximal at 10 and 3 min, respectively, and were still apparent at 30 min (Fig. 2). Although the maximal amount of AQP5 in the APM of the tissues treated with SNI-2011 was 70% of that for the tissues exposed to ACh (Fig. 2), the most marked difference between the effects of the full and partial muscarinic agonists was in the duration of the response.

Treatment of the tissues with U73122, TMB-8 or dantrolene completely inhibited the SNI-2011-induced increase in the amount of AQP5 in the APM (Table 1). These observations indicate that the interaction of SNI-2011 with M_3 AChRs activates PLC and subsequently increases the release of Ca^{2+} from intracellular stores via the activation of IP_3 receptors and RyRs. It has been proposed that an initial release of Ca^{2+} from the IP_3-sensitive pool acts as a primer for further Ca^{2+} release from the Ry-sensitive pool producing oscillations [22]. The characteristics of $[Ca^{2+}]_i$ oscillations generated by

full muscarinic agonists differ from those induced by partial agonists. ACh evoked a transient elevation of $[Ca^{2+}]_i$ lasting about 2–5 min [27]. Furthermore, the concentration of IP_3 is four-fold higher than that of control at 15 s after addition of ACh. In contrast, it is three-fold higher than that of control at 10 min after the addition of SNI-2011 (Fig. 5).

Collectively, these results indicate that SNI-2011 acting at M_3 AChRs in rat parotid acinar cells induces persistent oscillations of $[Ca^{2+}]_i$ and leads to long-lasting increases in the amount of AQP5 in the APM.

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