Changes in functioning of rat submandibular salivary gland under streptozotocin-induced diabetes are associated with alterations of Ca\(^{2+}\) signaling and Ca\(^{2+}\) transporting pumps

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Abstract

Xerostomia and pathological thirst are troublesome complications of diabetes mellitus associated with impaired functioning of salivary glands; however, their cellular mechanisms are not yet determined. Isolated acinar cells were loaded with Ca\(^{2+}\) indicators fura-2/AM for measuring cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) or mag-fura-2/AM—inside the endoplasmic reticulum (ER). We found a dramatic decrease in pilocarpine-stimulated saliva flow, protein content and amylase activity in rats after 6 weeks of diabetes vs. healthy animals. This was accompanied with rise in resting [Ca\(^{2+}\)]\(_i\), and increased potency of acetylcholine (ACh) and carbachol (CCh) but not norepinephrine (NE) to induce [Ca\(^{2+}\)], transients in acinar cells from diabetic animals. However, [Ca\(^{2+}\)], transients mediated by Ca\(^{2+}\) release from ER stores (induced by application of either ACh, CCh, NE, or ionomycin in Ca\(^{2+}\)-free extracellular medium) were decreased under diabetes. Application of inositol-1,4,5-trisphosphate led to smaller Ca\(^{2+}\) release from ER under the diabetes. Both plasmalemma and ER Ca\(^{2+}\)-ATPases activity was reduced and the latter showed the increased affinity to ATP under the diabetes. We conclude that the diabetes caused impairment of salivary cells functions that, on the cellular level, associates with Ca\(^{2+}\) overload, increased Ca\(^{2+}\)-mobilizing ability of muscarinic but not adrenergic receptors, decreased Ca\(^{2+}\)-ATPases activity and ER Ca\(^{2+}\) content.

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1. Introduction

Pathological thirst (polydipsia) is one of the troublesome complications of diabetes mellitus. Xerostomia (oral dryness) is a common complaint among diabetic patients, which is closely connected to polydipsia. Xerostomia is associated with damage to salivary gland activity, resulting in marked decrease in their ability to synthesize, transport and secrete saliva [1–3]. Effects of experimental diabetes induced by alloxan and streptozotocin (STZ) on rat salivary glands have been also documented. These reports suggest that diabetes caused an extensive cellular degeneration and structural and functional disorders [4–6]. At the same time, the intracellular mechanisms responsible for these changes are not yet clear.

The principal regulatory factors controlling the extent and nature of submandibular salivary gland secretions are autonomic neurotransmitters released by innervating sympathetic (norepinephrine) and parasympathetic nerves (acetylcholine). Acetylcholine (ACh) activates M\(_1\) and M\(_3\) muscarinic receptors, which are the only ACh-sensitive receptors found in the membrane of submandibular salivary gland cells [7]. Activation of both M\(_1\) and M\(_3\) receptors increases free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) via production of inositol-1,4,5-
trisphosphate (InsP3) and subsequent Ca\(^{2+}\) release by activation of InsP3 receptors of endoplasmic reticulum (ER). The recent availability of mutant mice deficient in specific mAChR subtypes enabled the demonstration of the prominent role of M\(_4\) receptors in cholinergic Ca\(^{2+}\) signaling, whereas the role of M\(_1\) receptors seems to be minor [8]. Norepinephrine (NE) activates both \(\alpha_1\) and \(\beta_1\) adrenergic receptors present in the submandibular acinar cells. Similar to muscarinic receptors, stimulation of \(\alpha_1\) adrenergic receptors in adult animals also leads to increase in InsP3 [9]. However the mechanism of \(\alpha\)-adrenergic activation of ryanodine receptors mediated by cyclic ADP-ribose was also proposed especially in younger animals [9].

Regulation of Ca\(^{2+}\) in the cytosol and ER lumen is central to the regulation of synthesis and secretion of saliva components [10,11], thus alteration of intracellular Ca\(^{2+}\) handling could be a basis for impaired salivary gland functioning associated with diabetes. To the best of our knowledge, there are no reports of altered [Ca\(^{2+}\)]\(_{\text{ER}}\), homeostasis in salivary cells, although this alteration is currently considered a basic pathology associated with diabetes complications. Numerous works originating from different laboratories testify that experimental diabetes leads to substantial changes in Ca\(^{2+}\) homeostasis in different cell types [12–16].

The ability of Ca\(^{2+}\) to function efficiently as an intracellular messenger is due to the existence of effective metabolic systems that keep [Ca\(^{2+}\)]\(_{\text{cyt}}\), at a low level in the cytosol and at a relatively high level in the lumen of the endoplasmic reticulum (ER). It is also important to note that the impairment of intrareticular Ca\(^{2+}\) handling could affect synthesis of secretory proteins, their chaperoning and maturation [17–19], reviewed in [11]. Regulation of Ca\(^{2+}\) in the cytosol and the ER is achieved by the concerted activities of several ion channels (some of them coupled to metabotropic receptors) and Ca\(^{2+}\)-ATPases localized in the endoplasmic reticulum (SERCA) and in the plasma membrane (PMCA). Numerous reports show that experimental diabetes alters Ca\(^{2+}\)-ATPase activity and expression in other tissues [20–26] but none pertain to salivary cells.

Thus, knowledge of abnormalities in Ca\(^{2+}\) signaling in the acinar secretory cells is fundamental to understanding the etiology of impaired salivation in diabetes. The present study examined this question.

2. Materials and methods

2.1. Induction of experimental diabetes

Male Wistar rats, 21 postnatal days old, weighting 30±5 g at the beginning of the experiment were used. They had free access to solid food and water in an animal room, which was maintained at 25 °C with a 12-h light/dark cycle. Diabetes was induced by a single intraperitoneal injection of streptozocin (STZ, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) dissolved in 0.9% NaCl solution using 60 mg/kg proportion; control animals were injected with saline alone. Animals were used for experiments 6–7 weeks after the onset of diabetes. All experiments were performed in accordance with regulations specified by Guide for the Care and Use of Laboratory Animals (1996, National Academy of Sciences, Washington D.C.) and approved by the Bogomoletz Institute of Physiology Clinic of Animal Care and Use.

2.2. Collection and analysis of saliva from submandibular gland

The rats were anesthetized with i.p. injection of mixture of 100 mg/kg body weight Ketamine (CuraMed Pharma GmbH, Karlsruhe, Germany) and 0.05 ml/kg body weight of Lysthenon (Nycomed GmbH, Austria) and then secured in supine position. Saliva was collected using modification of the cannulation method. Extra care was taken to prevent contamination of the submandibular saliva from other types of saliva, for that the opening of the tapered end of a glass cannula was chosen to resemble the size of submandibular duct papilla, therefore providing its tight coverage. The other end of the cannula was connected via a variable speed peristaltic pump to a pre-weighted 1.5 ml centrifuge tube for collecting the secreted saliva. Pilocarpine (2 mg/kg, i.p.) was used to stimulate the salivation and saliva was collected for 10 min after the injection. Saliva protein content was determined using Lowry method, amylase activity by Caraway method using amylase-test kit (Biotron diagnostics, Hemet, CA, USA).

2.3. Isolation of submandibular salivary gland’s acinar cells

Submandibular salivary gland acinar cells were isolated as described previously [27] using digestion with collagenase P and mechanical dissociation with Pasteur pipettes. Suspension was filtered through 70 μm cell strainer to get acini of approximately same size and after that in the Tyrode solution. Tyrode solution contained (in mM): NaCl—135, KCl—5, HEPES—10, MgCl\(_2\)—2, CaCl\(_2\)—2, glucose—10 (pH 7.35). To obtain a Ca\(^{2+}\)-free solution, CaCl\(_2\) was omitted, MgCl\(_2\) was increased to 4 mM and 1 mM EGTA was added.

2.4. Fluorescence imaging of [Ca\(^{2+}\)]\(_{\text{cyt}}\), and [Ca\(^{2+}\)]\(_{\text{ER}}\)

For [Ca\(^{2+}\)]\(_{\text{cyt}}\), measurements, cells were loaded with Ca\(^{2+}\) indicator fura-2/AM (10 μM) for 35 min at 35 °C. Fura-2 ratios were captured at 5 frames per second as described elsewhere [13]. [Ca\(^{2+}\)]\(_{\text{ER}}\) values were calculated online using Grynkiewicz equation [28]. For [Ca\(^{2+}\)]\(_{\text{ER}}\) measurements, mag-fura-2 was loaded into cells of intact salivary glands by a 45-min incubation with 6 μM mag-fura-2/AM and 0.02% pluronic F-127 at 37 °C in Tyrode solution. After mounting in the superfusion chamber, cells were briefly washed with intracellular-like media (ICM) and permeabilized for 4–8 min with 40 μg ml\(^{-1}\) β-escin in ICM. Permeabilization was confirmed by monitoring the fall of mag-fura-2 fluorescence (excited at 360 and 390 nm and detected at 510 nm) to less than 20% of its initial intensity. Changes in [Ca\(^{2+}\)]\(_{\text{ER}}\) are expressed as changes in the ratio of mag-fura-2/AM fluorescence at 360 nm to 390 nm (F\(_{\text{360/F390}}\) that are proportional to [Ca\(^{2+}\)]\(_{\text{ER}}\). The ICM contained (in mM): KCl—120; NaCl—20; MgSO\(_4\)—2; HEPES—10; ATP—5; EGTA—1; CaCl\(_2—0.75\) (pH 7.2).

2.5. Microsomal preparation

All procedures were done at 4 °C using precooled reagents. Immediately after removal from the body, gland tissue was washed and homogenized at 4 °C in ice cold solution containing (in mM): sucrose—250, EDTA—1, Tris—HCl—10 (pH 7.4) using 10–15 strokes of a motor-driven glass-tellon homogenizer. Homogenate was centrifuged at 1600×g for 10 min to remove nuclear fraction. Supernatant was then centrifuged at 10,000×g for 10 min to remove pelleted mitochondria. Resulting supernatant was centrifuged at 100,000×g for 40 min to yield microsomal pellet. The resulting microsome pellet was resuspended in homogenization buffer. Microsomes were either used immediately or stored frozen at −40 °C.

2.6. ATPase assay

A reaction buffer contained (in mM): NaCl—50, KCl—100, Tris—HCl—20, MgCl\(_2\)—3, CaCl\(_2—0.01\) (pH 7.4; T=37 °C); samples contained 3–5 μg/ml of protein. The reaction was initiated by the addition of 3 mM ATP at 37 °C and terminated 1.5 min after initiation by the addition of 20% ice-cold trichloracetic acid. The time was set to be within the linear range of the reaction. Ca\(^{2+}\)-ATPase activity was measured by colorimetric determination of inorganic phosphate (P\(_i\)) liberated from ATP using a previously described method [29]. To exclude a possible mitochondrial contaminant, sodium azide (1 mM) was added to the
incubation medium. Ouabain (1 mM) was added to the incubation medium to suppress plasma membrane Na+/K+-ATPase activity. The Ca\(^{2+}\)-independent ATPase activity was assayed in the presence of 5 mM EGTA without the addition of Ca\(^{2+}\). The Ca\(^{2+}\)-dependent ATPase activity (Ca\(^{2+}\)-ATPase activity) was obtained by subtracting the Ca\(^{2+}\)-independent ATPase activity from the total activity. To evaluate the activity of SERCA thapsigargin (500 nM) was used. SERCA activity was calculated from the difference between total Ca\(^{2+}\)-ATPase activity and a thapsigargin-insensitive fraction of Ca\(^{2+}\)-ATPase activity (PMCA activity).

2.7. Data analysis

Ca\(^{2+}\) recording traces were analyzed using Axon imaging workbench software (Axon Instruments, Union City, CA, USA). Data are expressed as mean±S.E.M. Equal variance analysis was performed between the control and STZ group. Statistical significance was calculated using paired and unpaired two-tailed Student’s t-test where appropriate; a P value of less than 0.05 was considered as statistically significant.

3. Results

Fourteen saline-control, 5 STZ-control and 15 diabetic rats were used in experiments devoted to studying diabetes-induced changes in Ca\(^{2+}\) signaling mechanisms. Animals for a STZ-control group were taken into experiments 1–2 days after STZ injection. We did not observe any difference either in salivation or in Ca\(^{2+}\) signaling between saline-control and STZ-control groups, indicating that STZ itself did not affect salivation and Ca\(^{2+}\) homeostasis in salivary cells. Diabetic rats were taken into experiments in 6–7 weeks after STZ injection. On average, the glucose concentration in blood serum was 117±18 mg/dl (n=14) and 432±90 mg/dl (n=15) for control and diabetic animals, respectively.

3.1. In vivo studies of submandibular salivary gland functioning under experimental diabetes

To test the changes in functioning of submandibular salivary gland under experimental diabetes, we studied the main parameters of stimulated salivation in healthy and diabetic rats in vivo. Since the body weight of diabetic animals had a substantial dispersion (mean 218 g, range 120–250g), we normalized saliva flow rate (i.e., volume of saliva secreted during 1 h) to the weight of the animal, as changes in body weight and submandibular gland weight parallel one another. Intraperitoneal injection of pilocarpine (a substance for stimulation of saliva secretion via activation of salivary cells’ muscarinic receptors) increased the salivary flow rate both in healthy and diabetic animals. Nevertheless, in diabetic animals the saliva flow rate after pilocarpine stimulation was significantly lower (43±8%, P<0.001, Table 1) than in control rats. Similar to saliva flow rate, we observed a significant decrease in saliva protein content of diabetic animals after pilocarpine stimulation compared to controls (24±4%, P<0.01, Table 1). We have also found a dramatic impairment of saliva amylase activity in diabetic animals that totaled 85±5% (P<0.001, Table 1) as compared to control animals.

Thus, we observed a dramatic decrease in submandibular gland saliva secretion, total saliva protein content and amylase activity under diabetes. Since regulation of both protein synthesis and secretion is strongly dependent on intracellular Ca\(^{2+}\), we studied the potential changes in intracellular Ca\(^{2+}\) signaling under experimental diabetes.

3.2. Impairment of Ca\(^{2+}\) homeostasis in salivary cells under experimental diabetes

3.2.1. Resting [Ca\(^{2+}\)]\(_{i}\), level

We observed a significant rise in resting [Ca\(^{2+}\)]\(_{i}\), in cells from diabetic animals. We found that resting intracellular [Ca\(^{2+}\)]\(_{i}\), in acinar cells isolated from control animals was 95±4 nM (n=66) and 152±3 nM (n=80) in cells from diabetic animals (Fig. 1D). The rise in resting [Ca\(^{2+}\)]\(_{i}\), level comprised 60±4% (P<0.001).

3.2.2. [Ca\(^{2+}\)]\(_{i}\), transients induced by ACh

We found that the amplitudes of [Ca\(^{2+}\)]\(_{i}\) transients induced by bath application of ACh (5 μM) in Ca\(^{2+}\)-containing Tyrode solution were significantly larger in submandibular acinar cells from diabetic rats (Fig. 1A, B). On average, the amplitudes of ACh-induced [Ca\(^{2+}\)]\(_{i}\) transients were 226±15 nM (n=65) and 278±13 nM (n=75) in cells from control and diabetic animals, respectively. The increase was significant (P<0.01) and totaled 23±3% (Fig. 1A, B). We found that the time constant (τ) of the decay phase of ACh-induced [Ca\(^{2+}\)]\(_{i}\) transients, determined by single-exponential fits, were significantly prolonged by 57±11% (P<0.01) in diabetic animals. On average, the τ values were 10.8±1.3 (n=40) and 17±1.5 (n=52) s in salivary cells of control and diabetic animals respectively. In order to quantify the altered sensitivity of ACh receptors, we measured the dose–response relationship in cells from control and diabetic animals. We found that under STZ-induced diabetes, the dose–response curve of ACh-evoked [Ca\(^{2+}\)]\(_{i}\), transients has a significant (P<0.01) leftward shift and elevated maximum compared to control (Fig. 1C). Effective concentration of ACh producing half-maximal response (EC50), calculated using a Boltzmann fit of experimental points, totaled 0.83±0.06 μM and 0.53±0.05 μM in control and diabetic conditions respectively.

3.2.3. [Ca\(^{2+}\)]\(_{i}\), transients induced by NE

NE was also able to elicit [Ca\(^{2+}\)]\(_{i}\), transients by activation of α\(_{1}\)-adrenoceptors, which is the only adrenoceptor subtype known to act through InsP\(_{3}\) pathway. We found that the amplitudes of [Ca\(^{2+}\)]\(_{i}\), transients induced by bath application of NE (10 μM) in Ca\(^{2+}\)-containing Tyrode solution were significantly smaller in submandibular acinar cells from diabetic

<table>
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<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetes</th>
</tr>
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<tr>
<td>Saliva flow rate, ml/kg/h</td>
<td>20.6±1.1 (n=14)</td>
<td>11.7±1.7 (n=10)*</td>
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<tr>
<td>Protein content, mg/ml</td>
<td>4.2±0.6 (n=14)</td>
<td>3.2±0.5 (n=10)*</td>
</tr>
<tr>
<td>Amylase activity, mg/sec/l</td>
<td>796±33 (n=9)</td>
<td>118±10 (n=10)*</td>
</tr>
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</table>

Values are mean±S.E.M. (number of rats). Statistical analysis was conducted by means of Student’s t-test. * P<0.01 diabetes vs. control.

Table 1 Parameters of salivation in healthy animals and rats with STZ-induced diabetes

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rats (Fig. 2A, B). On average, the amplitudes of NE-induced [Ca^{2+}]_i transients were 65±11 nM (n=10) and 42±9 nM (n=8) in cells from control and diabetic animals respectively. The decrease was significant (P<0.01) and totaled 32±5% (Fig. 2C).

### 3.2.4. Ca^{2+} release from InsP_{3}-sensitive Ca^{2+} store

To study the diabetes-induced changes of inositol-1,4,5-trisphosphate (InsP_{3})-sensitive Ca^{2+} release from the ER, we used agonists of mAChRs: ACh and its hydrolysis-resistant analogue carbamylcholine (CCh). Application of ACh and CCh was performed in Ca^{2+}-free extracellular medium to exclude involvement of external Ca^{2+} influx. Application of ACh (5 μM) in Ca^{2+}-free extracellular solution led to a transient [Ca^{2+}]_i rise with the average amplitude of 158±14 nM (n=19) for control and 135±11 nM (n=21) for diabetic animals (Fig. 1A, B). Hence, we observed a significant (14±4%, P<0.05) decrease in the amplitude of [Ca^{2+}]_i release from the ER upon activation of mAChRs. The ratio of the amplitude of ACh-induced [Ca^{2+}]_i transients in Ca^{2+}-free media to that in Ca^{2+}-containing solutions was 0.68±0.03 (n=19) for control and 0.49±0.03 (n=21) for diabetic animals (Fig. 1D).

We also found a significant decrease in the amount of releasable Ca^{2+} from InsP_{3}-sensitive Ca^{2+} stores upon activation of mAChRs by CCh in salivary cells of diabetic animals. An application of CCh (5 μM) in Ca^{2+}-free extracellular solution led to a transient [Ca^{2+}]_i rise with the average amplitude of 142±15 nM (n=18) for control and 96±6 nM (n=35) for diabetic animals (Fig. 3A, B). This decrease was significant (P<0.001) and comprised 32±3%. The dose–response curve of CCh-evoked [Ca^{2+}]_i transients also showed a significantly (P<0.01) increased sensitivity (decreased EC50 value) to CCh, while the CCh curve had a significantly (P<0.01) decreased slope and maximum value under STZ-induced diabetes (Fig. 3C). CCh EC50 and slope values were 4.7±0.6 μM and 0.9±0.04 in cells from control animals and 3.0±0.5 μM and 0.6±0.03 in cells from diabetic rats, respectively.
Similar to activation of mAChRs with application of ACh and CCh in Ca²⁺-free extracellular solution, activation of α₁-
adrenoceptors with NE also led to significantly decreased Ca²⁺ release from InsP₃-sensitive Ca²⁺ stores in acinar cells from diabetic animals. On average, application of NE (10 μM) in Ca²⁺-free extracellular solution led to a transient [Ca²⁺]ᵢ rise with the amplitude of 37±9 nM (n=10) and 25±8 nM (n=8) for control and diabetic conditions correspondingly (Fig. 2A, B). Hence, we observed a significant (33 ± 7%, P<0.05) decrease in the amplitude of [Ca²⁺]ᵢ release from the ER upon activation of α₁-adrenoceptors.

3.2.5. Ca²⁺ release from the ER by ionomycin

To test the changes in the amount of Ca²⁺ stored within the ER, we used ionomycin (Ca²⁺ ionophore that, in the absence of external Ca²⁺, releases Ca²⁺ from the ER in receptor-independent manner [30]). We found that Ca²⁺ liberation from ER stores induced by ionomycin was significantly decreased in diabetes. On average, amplitudes of ionomycin (500 nM)-induced Ca²⁺ release were 95±12 nM (n=10) in control and 47±7 nM (n=16) in diabetic conditions, a significant 51±5% decrease (P<0.01, Fig. 3D).

3.2.6. Ca²⁺ release from the ER by InsP₃

To directly access changes in the amount of Ca²⁺ released from the ER upon activation of InsP₃ receptors, we loaded cells with low-affinity Ca²⁺ indicator—mag-fura-2/AM, which after plasma membrane permeabilization remains trapped inside the ER lumen. We found that the application of InsP₃ (3 μM) to permeabilized acinar cells produced transient decrease in the ratio of F₃60/F₃90 that is proportional to the decrease in Ca²⁺ concentration inside the ER. The magnitude of the decay in the F₃60/F₃90 ratio was 0.037±0.004 (n=10) and 0.021±0.005 (n=14), in cells from control and diabetic animals respectively (Fig. 3E). This reduction was significant (P<0.01) and comprised 47±10% comparing to control conditions. We also...
found that the time constants ($\tau$) of the $[Ca^{2+}]_{ER}$ recovery phase after the application of InsP$_3$ and subsequent re-addition of ATP (3 mM), which reflects the rate of the ER $Ca^{2+}$ store refilling by SERCA, were significantly prolonged by 129±17.5% ($P<0.05$) in diabetic animals. On average, the $\tau$ values were 16±2 s ($n=10$) and 37±5 s ($n=14$) s in salivary cells of control and diabetic animals respectively (Fig. 3F).

Thus, we observed that the amplitudes of $Ca^{2+}$ transients due to $Ca^{2+}$ release from the InsP$_3$- and ionomycin-sensitive intracellular $Ca^{2+}$ stores of the ER are significantly diminished
under diabetic conditions. We can suggest that Ca\(^{2+}\) extrusion from the cytosol to the extracellular medium may also be deteriorated under diabetes, considering the increased resting [Ca\(^{2+}\)\(_c\)], and the substantial prolongation of ACh- and InsP\(_3\)-induced Ca\(^{2+}\) signals. These findings may be due to dysfunction of SERCA and/or PMCA, which are responsible for the accumulation of Ca\(^{2+}\) in the ER and its extrusion from the cytosol. To directly test this hypothesis, we assayed the specific activities of PMCA and SERCA in control and diabetic conditions.

3.3. Changes in activity and regulation of Ca\(^{2+}\)-ATPases under experimental diabetes

Specific activities of Ca\(^{2+}\)-ATPases were measured in tissue from 28 control and 24 diabetic rats and divided into 7 and 6 samples respectively.

3.3.1. Kinetic parameters of total Ca\(^{2+}\)-dependent ATP hydrolysis

We found significant changes in kinetics of Ca\(^{2+}\)-dependent ATP hydrolysis in salivary cells under STZ-induced diabetes (Table 2). On average, the maximal amount of inorganic phosphate (P\(_i\)) liberated during Ca\(^{2+}\)-dependent ATP-hydrolysis in the presence of saturating ATP concentration (P\(_{max}\)) was decreased by 52±6% (P<0.01). The initial activity of the P\(_i\) liberation (V\(_{max}\)) was diminished by 50±7% (P<0.01) under experimentally-induced diabetes, whereas the time course of activity (the time needed to liberate the amount of P\(_i\)=P\(_{max}/2\)) was not significantly different.

3.3.2. ATP dependence of Ca\(^{2+}\)-ATPase functioning in control and STZ-induced diabetes

The ATP dependence of the both PMCA and SERCA was examined at 0.01–10 mM ATP and [Ca\(^{2+}\)\(_o\)]\(_c\)~1 μM. Under the STZ-induced diabetes maximal activities (V\(_{max}\)) of both Ca\(^{2+}\)-ATPases appear to be reduced by 47±9% (P<0.01) for PMCA and 79±8% (P<0.01) for SERCA (Fig. 4, Table 2). The Hill plots of ATP dependence of the Ca\(^{2+}\)-ATPase activities in diabetes exhibited a significant 73±11% (P<0.01) decrease in apparent Michaelis constant (K\(_m\)) for ATP for SERCA and no significant change for PMCA (Fig. 4, Table 2). The ATP dependence of PMCA and SERCA exhibited a non-cooperative profile with n\(_H\)~1.0 both in control and diabetic conditions.

3.3.3. Ca\(^{2+}\) dependence of Ca\(^{2+}\)-ATPase functions in control and STZ-induced diabetes

The Ca\(^{2+}\) dependence of the Ca\(^{2+}\)-ATPase activities (both PMCA and SERCA) was examined at [Ca\(^{2+}\)]\(_c\)~0.007–300 μM and 3 mM ATP. The decrease in V\(_{max}\) under STZ-diabetes was similar to that observed in the experiments with ATP and totaled 46±7% (P<0.01) and 70±11% (P<0.01) for PMCA and SERCA correspondingly (Table 2). We did not find a significant difference in K\(_m\) and Hill coefficient of the Ca\(^{2+}\) dependence of PMCA and SERCA molecules between control and diabetic conditions (Table 2).

4. Discussion

In this study we examined the effects of STZ-induced diabetes on main parameters of salivation in response to muscarinic receptor stimulation in the rat submandibular salivary gland. Since submandibular gland is believed to be the main source of basal whole saliva, impairment of its functioning could largely contribute to the development of xerostomia. The human studies also suggest impairment of submandibular but not parotid gland in Type II diabetic patients [1]. We found a marked decrease in saliva flow, protein content and amylase activity in diabetic animals under pilocarpine stimulation. This is consistent with the findings observed at the early stages (2–4 weeks) of STZ-induced diabetes in rat where prominent decrease in saliva flow under pilocarpine stimulation was shown [31,32]. However at long terms (6–12 months) of diabetes development there is no indication of differences in saliva flow either from pilocarpine or parasympathetically stimulated glands [33,34], indicating the availability of some compensatory mechanisms. Nevertheless, the cellular mechanisms

Table 2

<table>
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<th>Parameter</th>
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<td>(V_{max}), μmol/h/mg</td>
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<td>(K_m) (ATP), mM</td>
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<tr>
<td>(V_{max}) (Ca(^{2+})), μmol/h/mg</td>
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<td>n(_H) (Ca(^{2+}))</td>
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Values are mean±S.E.M. Statistical analysis was conducted by means of Student’s t-test.

* P<0.01 diabetes vs. control.
explaining decreased salivation and protein concentration as well as compensatory changes under diabetes are not clear.

Cytoplasmic and luminal Ca²⁺ signaling regulate secretion of saliva components as well as their synthesis [10,18,19] and could play an important role in the observed changes in salivation under experimental diabetes. We indeed found dramatic changes in Ca²⁺ homeostasis and signaling in acinar cells of rat submandibular salivary gland after 6–7 weeks of diabetes development. First, we found elevated resting [Ca²⁺]i levels and increased potency of ACh and CCh to induce [Ca²⁺]i transients. On the other hand, we observed a reduction in amplitudes of all [Ca²⁺]i responses mediated by liberation of Ca²⁺ solely from the endoplasmic reticulum store ([Ca²⁺]i transients evoked by either ACh, CCh, NE or ionomycin in Ca²⁺-free medium). We believe that there is a superimposition of several mechanisms; first is the increased Ca²⁺-mobilizing ability of muscarinic receptors, second is the increased Ca²⁺ influx across plasma membrane, and third is the diminished Ca²⁺ content inside the ER lumen.

The assertion of the increased Ca²⁺-mobilizing ability of muscarinic receptors under experimental diabetes is supported by (i) decreased EC50 values for both ACh and CCh; (ii) potentiation of ACh-induced [Ca²⁺]i transients. Increased potency of muscarinic receptors under experimental diabetes had not previously been shown for submandibular salivary gland, however, this effect is quite common in other tissues [35–39] and, as speculated, could be an intrinsic protector mechanism to the development of autonomic neuropathy causing a decreased tone of the autonomic nervous system, which is shown in both rat and human studies [2,40–42]. Nevertheless, the increased amplitude of ACh-induced [Ca²⁺]i transients in Ca²⁺ containing extracellular solution cannot be explained only by increased sensitivity of muscarinic receptors, since mAChR-mediated Ca²⁺ release from the ER is reduced. That means an increased Ca²⁺ influx across plasma membrane, though the molecular mechanism has not been explored. It is generally believed that the major source of transmembrane Ca²⁺ influx is the Ca²⁺ release-
activated Ca$^{2+}$ (CRAC) channels [10,43]. There is also evidence of a store-independent G-protein coupled transmembrane Ca$^{2+}$ channel [44] in submandibular salivary cells, presumably transient receptor potential-like protein 6 (TRP6), coupled via G-protein to M$_1$ and M$_3$ receptors [45]. We cannot point out what particular mechanism is involved in the increased Ca$^{2+}$ influx under experimental diabetes, but it is likely that increased sensitivity of mAChRs can lead to increased activation of G-protein coupled Ca$^{2+}$ channels like TRP6.

Magnitudes of NE-induced [Ca$^{2+}$]$_i$ transients were much smaller that those induced by ACh, which is not surprising because NE-induced increase in the InsP$_3$ levels is several fold less than that induced by ACh [9]. On the contrary to ACh, NE-evoked [Ca$^{2+}$]$_i$ transients in Ca$^{2+}$-containing extracellular solution were decreased during diabetes indicating that potentiation of Ca$^{2+}$ responses induced via activation of muscarinic receptors is not common for other receptor subtypes. Impairment of Ca$^{2+}$ signaling induced by NE is consistent with in vivo experiments, which showed decreased submandibular salivary flow in response to adrenergic stimulation and reduced protein concentration in submandibular saliva during symaptetic stimulation [31,33,46].

Despite the potentiation of ACh-induced [Ca$^{2+}$]$_i$ transients in Ca$^{2+}$-containing medium, the amplitudes of all ACh-, CCh- and NE-induced [Ca$^{2+}$]$_i$ transients in Ca$^{2+}$-free solution were decreased under diabetes. Application of InsP$_3$ also induced smaller release of Ca$^{2+}$ from the ER indicating that either level of [Ca$^{2+}$]$_{ER}$ is lowered or sensitivity of InsP$_3$ receptors is changed under diabetes. Furthermore, we observed the reduction in magnitude of Ca$^{2+}$ release from the ER independently of activation of InsP$_3$ receptors, i.e., with ionotocyn that further supports the hypothesis of decreased Ca$^{2+}$ content inside ER lumen. Interestingly, the reduction in Ca$^{2+}$ release by ionomycin was more pronounced than via InsP$_3$ receptors driven by mAChR activation, further supporting the idea of increased sensitivity of muscarinic receptors under diabetes.

Along with reduced Ca$^{2+}$ content of the ER we observed an increased resting [Ca$^{2+}$]$_i$ and delayed clearance of ACh- and InsP$_3$-induced Ca$^{2+}$ signals. This may reflect altered functioning of cells’ Ca$^{2+}$-transporting systems like SERCA and PMCA. Moreover slower replenishment of [Ca$^{2+}$]$_{ER}$ after the application of InsP$_3$ clearly indicate deteriorated functioning of SERCA since in given experimental conditions it is the only system responsible for the ER refilling. We show, for the first time, that STZ diabetes dramatically reduces the Ca$^{2+}$ transporting ability of both PMCA and SERCA from rat submandibular gland, which may explain both increased resting [Ca$^{2+}$]$_i$ and diminished ER Ca$^{2+}$ content. This could be due to the diminished number of rotations of the enzyme molecule as a result of increased lipid peroxidation [25] and/or altered conformational transitions within E1–E2 model caused by non-enzymatic glycation of the protein molecule [47]. However, other mechanisms are likely involved, as non-enzymatic glycation cannot account for the elevated sensitivity of SERCA molecule to ATP [47] or explain the more pronounced suppression of SERCA activity over PMCA. One of the plausible mechanisms is an increased expression of phospholamban (PLB) under diabetes [24], the ancillary inhibitory protein whose detachment exposes the high-affinity Ca$^{2+}$ binding sites of SERCA molecule. However, a recent report suggests that an increase in PLB may not fully account for the diabetes-induced impairment of SERCA and introduces a novel mechanism of its postranslational modification during diabetes [48].

We showed in this report that impairment of salivary cells functioning under the diabetes is associated with the changes in Ca$^{2+}$ signaling. Decreased activities of Ca$^{2+}$-ATPases and increased Ca$^{2+}$ influx appears to be related to increased [Ca$^{2+}$]$_i$, a condition referred to as “Ca$^{2+}$ overload”. Long time Ca$^{2+}$ overload may lead to Ca$^{2+}$ accumulation in the mitochondria followed by ATP depletion and thereby adverse effects on cellular functioning [49]. Decreased activity of SERCA may account for the decreased ER Ca$^{2+}$ refilling that, in turn, leads to diminished Ca$^{2+}$ content inside the ER lumen. The latter results in improper posttranslational processing of ER protein [17], folding and compromised exit of proteins [18,19] that could lead to the observed decrease in saliva protein content and amylase activity.

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