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Distinct Mechanisms of $[Ca^{2+}]_i$ Oscillations in HSY and HSG Cells: Role of Ca^{2+} Influx and Internal Ca^{2+} Store Recycling

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Abstract. This study examined $[Ca^{2+}]_i$ oscillations in the human salivary gland cell lines, HSY and HSG. Relatively low concentrations of carbachol (CCh) induced oscillatory, and higher [CCh] induced sustained, steady-state increases in $[Ca^{2+}]_i$ and K_{Ca} currents in both cell types. Low IP_3, but not thapsigargin (Tg), induced $[Ca^{2+}]_i$ oscillations, whereas Tg blocked CCh-stimulated $[Ca^{2+}]_i$ oscillations in both cell types. Unlike in HSG cells, removal of extracellular Ca^{2+} from HSY cells (i) did not affect CCh-stimulated $[Ca^{2+}]_i$ oscillations or internal Ca^{2+} store refill, and (ii) converted high [CCh]-induced steady-state increase in $[Ca^{2+}]_i$ into oscillations. CCh- or thapsigargin-induced Ca^{2+} influx was higher in HSY, than in HSG, cells. Importantly, HSY cells displayed relatively higher levels of sarcoendoplasmic reticulum Ca^{2+} pump (SERCA) and inositoltrisphosphate receptors (IP_3Rs) than HSG cells.

These data demonstrate that $[Ca^{2+}]_i$ oscillations in both HSY and HSG cells are primarily determined by the uptake of Ca^{2+} from, and release of Ca^{2+} into, the cytosol by the SERCA and IP_3R activities, respectively. In HSY cells, Ca^{2+} influx does not acutely contribute to this process, although it determines the steady-state increase in $[Ca^{2+}]_i$. In HSG cells, $[Ca^{2+}]_i$ oscillations directly depend on Ca^{2+} influx; Ca^{2+} coming into the cell is rapidly taken up into the store and then released into the cytosol. We suggest that the differences in the mechanism of $[Ca^{2+}]_i$ oscillations HSY and HSG cells is related to their respective abilities to recycle internal Ca^{2+} stores.

Key words: Ca^{2+}-activated K^+ channel — $[Ca^{2+}]_i$ oscillations — Salivary gland cell lines — Ca^{2+} store — SERCA — IP_3R

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Introduction

Activation of muscarinic receptors on the plasma membrane of salivary and other exocrine gland cells leads to stimulation of the inositol phosphate signaling pathway. The resulting increase in intracellular inositol 1,4,5 trisphosphate (IP_3) induces an increase in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$); due to IP_3-dependent release of Ca^{2+} from internal Ca^{2+} stores via the IP_3 receptor (IP_3R) [1–3]. Oscillatory increases in $[Ca^{2+}]_i$ have been reported in many nonexcitable cells, including those from exocrine glands [1–4, 6–8, 19–21, 23]. The simplest mechanism proposed to describe $[Ca^{2+}]_i$ oscillations involves an increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores (upstroke) and decrease in $[Ca^{2+}]_i$ due to uptake of Ca^{2+} into the stores, or efflux from the cell, (downstroke) following inactivation (peak) of the release pathway. Oscillations of $[Ca^{2+}]_i$ have been suggested to be generated by the biphasic effect of $[Ca^{2+}]_i$ on the IP_3 receptor; for example, low $[Ca^{2+}]_i$ promotes but high $[Ca^{2+}]_i$ inhibits IP_3-induced Ca^{2+} release. In addition, the $[Ca^{2+}]$ in the lumen of the Ca^{2+} store has also been suggested to regulate the permeability of the IP_3 receptor. In general, there are two main types of oscillatory patterns, (i) base-line spikes in which $[Ca^{2+}]_i$ oscillates from a baseline which is close to the resting $[Ca^{2+}]_i$, (ii) oscillations superimposed on a sustained steady-state elevation in $[Ca^{2+}]_i$ [3]. Both kinds of oscillations can be seen in exocrine gland cells such as pancreatic and salivary acinar cells [5, 8, 19–21]. We have reported that both types of oscillations occur in salivary epithelial cells [14]. Typically, very low concentrations of agonists produce base-line oscillations while higher agonist concentrations induce a sustained steady-state elevation of $[Ca^{2+}]_i$ that appears to be superimposed on the oscillations.

Intracellular Ca^{2+} release in nonexcitable cells is associated with the activation of the Ca^{2+} influx via a capacitative Ca^{2+} entry mechanism [1, 2, 20, 21]. Thus, the mechanism of $[Ca^{2+}]_i$ oscillations could be more complicated than discussed above. For example, $[Ca^{2+}]_i$ elevation due to Ca^{2+} influx could alter the amplitude or frequency of the oscillations [2]. Alternatively, sustained $[Ca^{2+}]_i$ oscillations could also display a complete dependence on the presence of external Ca^{2+}, i.e., on Ca^{2+} influx [8, 14]. $[Ca^{2+}]_i$ oscillations induced by low concentrations of certain agonists in pancreatic acinar cells do not appear to be accompanied by the stimulation of Ca^{2+} influx [19, 20]. For example, localized "spikes" of $[Ca^{2+}]_i$ have been seen at low concentrations of acetylcholine. These spikes appear to be independent of external $[Ca^{2+}]$ and Ca^{2+} influx. Possible explanations that have been suggested are that either the amount of Ca^{2+} released is not sufficient to stimulate Ca^{2+} influx or that specific store(s) within cells are depleted that are not associated with stimulation of Ca^{2+} influx. Sustained "global" oscillations or $[Ca^{2+}]_i$ waves have been shown to occur at higher concentrations of agonists and they are completely dependent on Ca^{2+} influx. Furthermore, in salivary gland cells, sustained $[Ca^{2+}]_i$ oscillations have been shown to be dependent on external Ca^{2+}, i.e., on Ca^{2+} influx [8, 14]. However, it is not clear whether this dependence of the oscillations on external Ca^{2+} is due to (i) depletion and refill of the internal store during each oscillation or (ii) direct regulation of the Ca^{2+} influx mechanism. In rat parotid acinar cells, slow oscillations in $[Ca^{2+}]_i$ have been reported to occur in the presence of thapsigargin and it has been suggested that they are a result of $[Ca^{2+}]_i$-dependent regulation of Ca^{2+} influx [8].

HSY cells, a human salivary ductal cell line of parotid origin, and HSG cells, a human salivary ductal cell line of submandibular origin, have been demonstrated to be useful experimental models to study the intracellular Ca^{2+} signaling mechanisms [10–16, 24–26]. Both cells demonstrate strong muscarinic receptor-coupled intracellular Ca^{2+} mobilization. In our previous study [14] we used the Ca^{2+}-activated K^+ channel (K_{Ca}) activity to examine the regulation of $[Ca^{2+}]_i$ oscillations in HSG cells. Our data demonstrated that sustained $[Ca^{2+}]_i$ oscillations in HSG cells are completely dependent on external Ca^{2+}. Further, we showed that the oscillations were likely due to the activation and inactivation of the IP_3 receptor, but that each oscillation involved Ca^{2+} influx-dependent refill of internal Ca^{2+} stores. Here we have examined CCh-stimulated oscillations in $[Ca^{2+}]_i$ in HSY cells. Our data demonstrate that the mechanism of $[Ca^{2+}]_i$ oscillations in HSY cells is distinct from that in HSG cells. We suggest that this difference is likely due to the relatively higher capacity of HSY cells to recycle the Ca^{2+} in the intracellular store(s).

Materials and Methods

Cell Culture

HSY and HSG cells were received from Dr. Mitsunobu Sato of the Second Department of Oral and Maxillofacial Surgery, Tokushima University, Japan, and were cultured in Eagle's minimum essential medium with Earl's balanced salt solution (EMEM, Biofluids, Rockville, MD). The culture medium was supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Biofluids) and changed three times a week. Cells were passaged when confluent. 0.25% trypsin-1.0 mM EDTA (Biofluids) was used for detaching cells from the tissue culture dish to yield a single-cell suspension. Then cells were reseeded in 35-mm culture dishes (Corning, NY) and grown for 24 hr before use.

Patch-Clamp Experiments

The electrophysiological methods used for this study has been described previously [14]. Briefly, the coverslips were placed in the perfusion chamber (Warner Instrument, Hamden, Connecticut) and the perfusion was achieved by a gravity-driven system. The bath solution was continuously removed through a vacuum line at a rate of approximately 5 ml/min. The pipette solution contained (mM) 150 KCl, 2 $MgCl_2$, 1 ATP, 5 HEPES, 0.1 EGTA, pH 7.2 (KOH). In some experiments 150 mM KCl was replaced with 150 mM CsCl and in others, 10 mM BAPTA, or 10–100 μM IP_3, was included in the pipette solution as indicated. The extracellular solution contained (mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 5 HEPES, pH 7.4 (NaOH).

The standard patch-clamp whole-cell technique was performed on single HSY and HSG cells attached to the coverslip at room temperature [9, 14, 15]. Patch electrodes were pulled from 1.0 mm borosilicate glass tubing using a Flaming/Brown micro-pipette puller (Sutter Instrument Co., Novato, CA). The resistance of the pipette was between 3–6 MΩ when filled with the standard pipette solution. An Ag-AgCl pellet was used to ground the chamber through a 150 mM NaCl-containing agar bridge. Whole-cell current was filtered at 2 KHz (low-pass bessel filter) with a sampling rate of 100 Hz and recorded directly onto the hard drive of a Dell Pentium computer at a holding potential of 0 mV. Membrane currents were amplified with an Axopatch 200A in conjunction with pClamp 6.1 software and a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA). Digitized data were analyzed using routines in pClamp 6.1 and Origin 5 (Microcal Software, Northampton, MA). The membrane potential was changed from −120 to +120 mV in 20 mV steps with 2.56 sec duration from a holding potential of −35 mV in the current-voltage relationship (*I-V*) experiments. The *I-V* relationship was calculated using the Clampfit module and exported to Origin 5 for further analysis. The mean K^+ current was calculated by dividing the total current with the time.

$[Ca^{2+}]_i$ Measurements

The fluorometric system used for intracellular Ca^{2+} measurements using indo-1 (Molecular Probes, Eugene, OR) has been described previously [12, 14]. The cells were loaded with indo-1 for 1 to 2 hr and then excited at 355 nm. The fluorescence emission at 410 and 485 nm was recorded using two photomultipliers simultaneously at room temperature. The output from each photomultiplier was sampled at a rate of 2 Hz. $[Ca^{2+}]_i$ was calculated by a customer-designed program using the F_{410}/F_{485} emission ratio and a calibration curve based on different

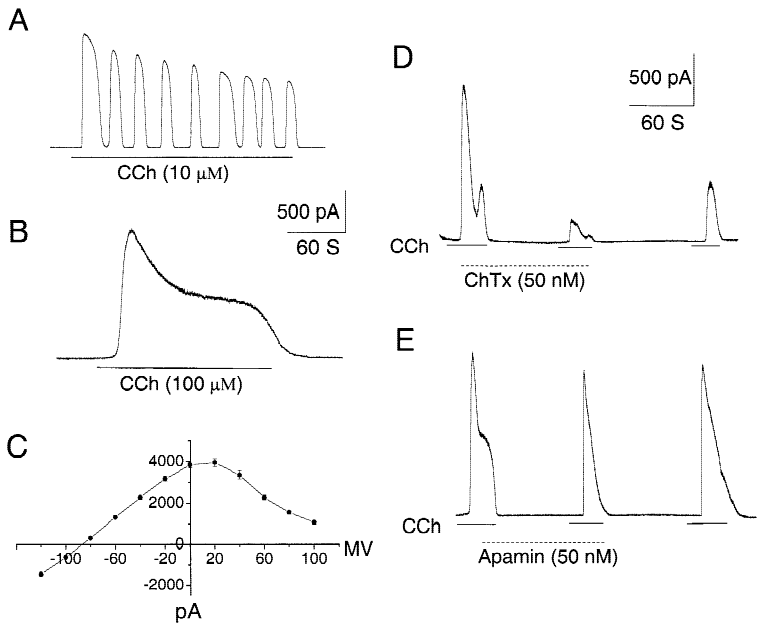

Fig. 1. CCh-stimulated outward K_{Ca} current in HSY cells. The current was recorded in single isolated HSY cells clamped at 0 mV. CCh and other agents were continuously perfused into the chamber at a rate of approximately 5 ml per min. 10 μM CCh (*A*) and 100 μM CCh (*B*) were applied to two different cells (traces are representative of results from more than thirty cells). (*C*) *I-V* relationship of the CCh-induced outward current, 10 μM CCh-induced peak current was plotted against the corresponding voltage (n = 5). ChTx (*D*) and apamin (*E*) were administered to two different cells for about three minutes after the first CCh-induced response. The cell was then restimulated with 100 μM CCh (30 sec duration). Representative of traces obtained with at least ten different cells are shown.

Ca^{2+} buffer solutions. The iris diaphragm was set to a field as small as a single cell so as to measure the fluorescence from one cell only. A similar perfusion system as that in patch-clamp experiments was used for application of different agents in the fluorescence study. Student's-t was used to test the statistical significance of the data. Data are expressed as mean ± SEM (standard deviation) and n equals to the number of the cells tested.

Preparation of HSG Cell Lysates

HSG cells, cultured as described above, were detached and suspended in ice-cold PBS containing 1% (v/v) aprotinin (Sigma, St. Louis, MO), centrifuged for 5 min at 900 × g, and resuspended in a lysis buffer containing (in mM): 100 Tris-HCl (pH 8.0), 1 $MgCl_2$, 0.5 4-(2-aminoethyl)-bezenesulfonyl fluoride hydrochloride (AEBSF) (ICN Biomedicals, Aurora, OH), 0.1 phenylmethylsulfonyl fluoride (PMSF) (Calbiochem, La Jolla, CA) and frozen at −80°C for at least two hours before use.

Preparation of Crude Membranes

HSG cell lysates (3–5 ml) were thawed on ice, homogenized in a Dounce homogenizer and diluted into sucrose buffer consisting of: 0.25 M sucrose, 10 mM Tris-HEPES (pH 7.4), 1% aprotinin (v/v), 1 mM dithiothreitol (DTT) (Calbiochem, Ultrol grade). The homogenate was centrifuged at 3000 × g for 15 minutes at 4°C and the resultant supernatant centrifuged at 50,000 × g for 30 min at 4°C. The pellet was resuspended in a minimal volume of the sucrose buffer and stored at −80°C until use. Protein concentration was determined by using the Biorad protein assay (microassay procedure).

SDS-PAGE and Western Blotting

Samples were diluted in a buffer containing 50 mM Tris-HCl (pH 8.8), 5 mM EDTA, and 1% SDS. Conditions for SDS-PAGE and Western blotting were as described previously [15]. PVDF membranes were blocked and incubated for 1 hr with a 1:1000 dilution of anti-IP_3R- or a 1:2500 dilution of the anti-SERCA2 antibody. The membrane was washed with TTBS, incubated as required with anti-mouse IgG, washed, treated with ECL reagents (Amersham Life Sciences, Piscataway, NJ), and exposed to X-OMAT™ films (Kodak).

Materials

All chemicals were ordered from Sigma (St. Louis, MO) except tert-butylhydroxyquinone (BHQ), Tg, apamin, choleratoxin (ChTx) and IP_3, which were from Calbiochem (La Jolla, CA), and BAPTA and indo-1, which were from Molecular Probes (Eugene, OR).

Results

Carbachol-Stimulation of K_{Ca} in HSY Cells

Activation of HSY cells with the muscarinic receptor agonist, carbachol (CCh), elicited an outward current that was measured using the standard whole-cell patch-clamp technique at a holding potential of 0 mV. Relatively low concentrations of CCh (1–10 μM) typically induced baseline-separated oscillatory increases in the outward current (Fig. 1*A*) while higher concentrations of CCh (>100 μM) induced a biphasic increase in the current, with an initial transient increase followed by a lower, sustained, steady-state increase (Fig. 1*B*). The *I-V* relationship of the CCh-induced outward current is shown in Fig. 1*C*. The reversal potential of the stimulated current was −85 mV (Fig. 2*C*), the K^+ equilibrium potential, suggesting that the current is carried by K^+

ions. Since K^+ channels have not yet been characterized in HSY cells, we examined this current further. Replacement of intracellular K^+ ions with Cs^+ totally abolished the CCh-induced outward current (*data not shown*, $n = 8$). Inclusion of 10 mM BAPTA in the pipette eliminated CCh-induced outward currents (*data not shown*, $n = 5$). Finally, ChTx, a large-conductance K_{Ca} channel inhibitor, reversibly blocked the CCh-induced outward current (Fig. 1*D*, upper trace), while apamin, a small-conductance K_{Ca} channel inhibitor, did not (Fig. 1*D*, lower trace). These data are similar to those reported for HSG cells and suggest that the two salivary gland cell lines have similar K_{Ca} channels. Furthermore, as in HSG cells, the K_{Ca} channel activity can be used as a readout for $[Ca^{2+}]_i$ [11, 14].

Involvement of Internal Ca^{2+} Stores in CCh-Stimulation of K_{Ca} in HSY Cells

CCh-induced activation of the K_{Ca} current was prevented by including BAPTA in the pipette solution (*data not shown*), suggesting that activation of the current depends on the increase of intracellular Ca^{2+}. Thus, we examined the effect of the intracellular Ca^{2+}-pump (SERCA) inhibitors, tert-butylhydroxyquinone (BHQ) and thapsigargin (Tg, *data not shown*), which inhibit Ca^{2+} uptake into the internal Ca^{2+} store(s) causing internal Ca^{2+} store-depletion, increase in $[Ca^{2+}]_i$, and activation of the store-operated Ca^{2+} influx [22]. BHQ induced an increase in the K_{Ca} current in HSY cells and blocked CCh-stimulation of the current (Fig. 2*A*). This suggests that BHQ and CCh mobilize Ca^{2+} from the same intracellular Ca^{2+} store. Furthermore, introduction of IP_3 into the cell via the patch pipette also increased the K_{Ca} current. More importantly, subsequent addition of CCh to the IP_3-stimulated cells failed to elicit further increases in K_{Ca} (Fig. 2*B*). Notably, ryanodine (0.1 μM) and caffeine (10 mM), which are widely used to study the Ca^{2+}-induced Ca^{2+} release, did not induce a significant increase in K_{Ca} current nor did either reagent affect the CCh-induced K_{Ca} response (Fig. 2 inset). These data indicate that CCh stimulation of the K_{Ca} current is mediated by an increase in $[Ca^{2+}]_i$ that is achieved via generation of IP_3 and release of Ca^{2+} from an IP_3- and BHQ-sensitive Ca^{2+} store. Thus, the IP_3-sensitive Ca^{2+} store plays a principal role in the initiation and the maintenance of CCh-induced oscillatory or sustained K_{Ca} currents in HSY cells.

Effect of Extracellular Ca^{2+} on the CCh-Stimulated K^+ Current and $[Ca^{2+}]_i$ Increases in HSY Cells

The effects of external Ca^{2+} on CCh-stimulated K_{Ca} current and $[Ca^{2+}]_i$ were examined. The sustained oscillations in K_{Ca} current induced by stimulation with low CCh (10 μM) were not altered by removal of external Ca^{2+} (Fig. 3*A*). Consistent with this, sustained oscillations in $[Ca^{2+}]_i$ seen at lower [CCh] were uninterrupted by the removal or re-addition of external Ca^{2+} (Fig. 3*B*). When the external Ca^{2+}-containing medium was replaced with Ca^{2+}-free medium, the sustained increase in K_{Ca} current, induced by 100 μM CCh, was converted to baseline oscillations that were maintained for a relatively long period (Fig. 4*A*). Reintroduction of Ca^{2+}-containing medium resulted in recovery of the sustained steady-state increase in the K^+ current. Consistent with this, the sustained $[Ca^{2+}]_i$ increase induced by the higher [CCh] was converted into an oscillatory increase when HSY cells were exposed to the Ca^{2+}-free external medium (Fig. 4*B*). In aggregate, the results presented in Figs. 3 and 4 show that low [CCh] stimulates sustained oscillations in $[Ca^{2+}]_i$ in HSY cells which are independent of external Ca^{2+}. At higher CCh concentrations the

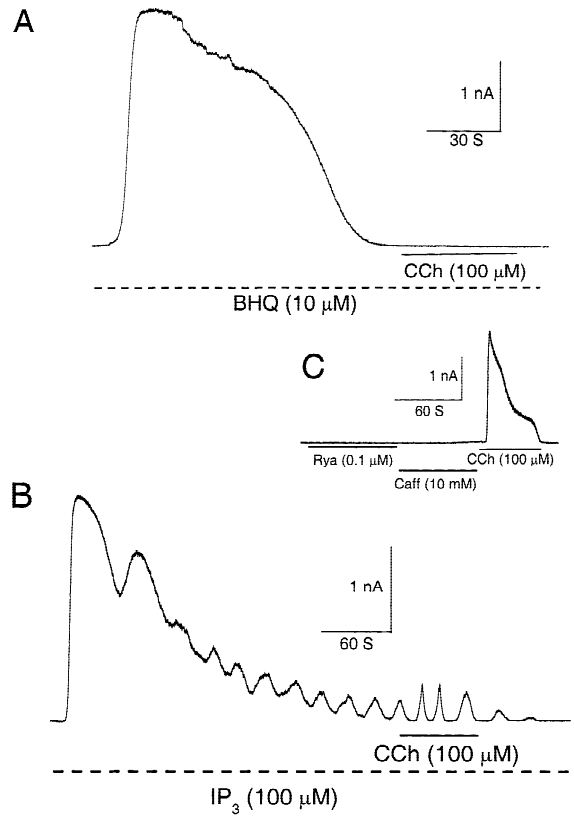

Fig. 2. Involvement of internal Ca^{2+} stores in the CCh-stimulated K^+ current. (*A*) BHQ (dashed line) and CCh (solid line) was applied to the cell. (*B*) IP_3 (100 μM) was directly introduced into the cytosol of the cell via the patch pipette (dashed line). The current was recorded as soon as the whole-cell configuration was established. CCh (100 μM) was added to the bath during the falling phase of IP_3-induced K^+ current (solid line). (*C*) (*Insert*) Effect of ryanodine and caffeine. These are traces representative of more than twenty cells.

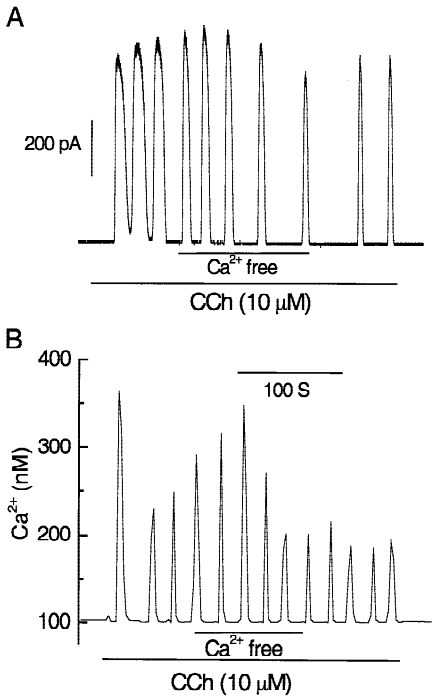

Fig. 3. Effect of external Ca^{2+} on CCh-stimulated oscillations in K_{Ca} and $[Ca^{2+}]_i$. Cells were stimulated with 10 μM CCh. K_{Ca} current (*A*) or $[Ca^{2+}]_i$ (*B*) were monitored as described in Materials and Methods. Ca^{2+}-containing perfusion medium was replaced with Ca^{2+}-free medium for the duration indicated in the figure.

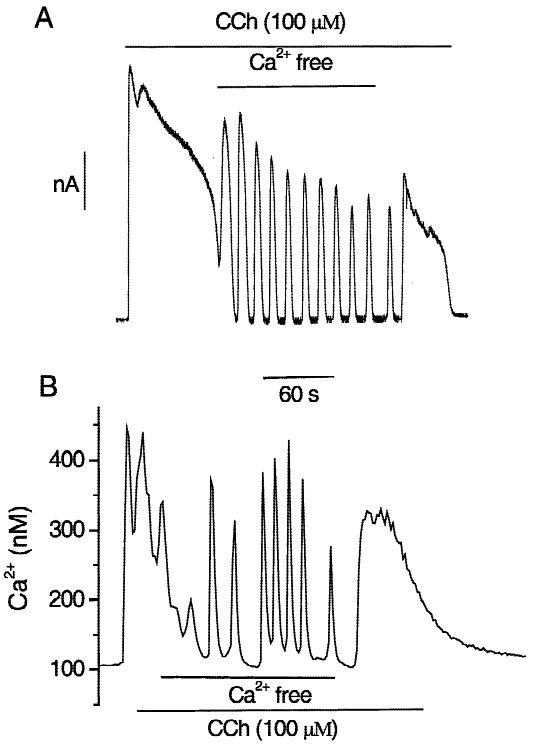

Fig. 4. Effect of external Ca^{2+} on sustained, steady-state elevations in K_{Ca} current and $[Ca^{2+}]_i$. K^+ current (*A*) or $[Ca^{2+}]_i$ (*B*) was measured in cells stimulated with 100 μM CCh. Ca^{2+}-containing external medium was replaced with Ca^{2+}-free medium for the indicated duration.

$[Ca^{2+}]_i$ increase appears to involve two components: (i) a sustained steady-state elevation that is dependent on external Ca^{2+} and superimposed on (ii), an oscillatory elevation in $[Ca^{2+}]_i$ that is independent of external Ca^{2+}. Similar oscillatory and sustained components of $[Ca^{2+}]_i$ increase were seen when high $[IP_3]$ was included in the pipette solution and external Ca^{2+} was removed (*data not shown*). This suggests that the external Ca^{2+}-independent $[Ca^{2+}]_i$ oscillations are not dependent on the concentration of IP_3. However, the oscillations are dependent on SERCA-mediated uptake of Ca^{2+} into the internal Ca^{2+} stores since addition of Tg to CCh-stimulated cells abolished oscillations in $[Ca^{2+}]_i$ (Fig. 5*A*) and K_{Ca} channel activity (*data not shown*).

The role of external Ca^{2+} in refilling of intracellular Ca^{2+} stores in HSY cells was examined. Fig. 5*B* shows that K_{Ca} current can be stimulated by repeated exposure of the cell to short pulses of 100 μM CCh. When external Ca^{2+} was removed during the first response and the cells were kept in this medium and exposed again to 100 μM CCh, the amplitude of the current induced was similar to that in cells maintained continuously in the Ca^{2+}-containing medium (Fig. 5*C*). These data suggest that internal Ca^{2+} stores in HSY cells can be refilled in the absence of external Ca^{2+}. This is consistent with the ability of HSY cells to undergo oscillations in $[Ca^{2+}]_i$ in the absence of external Ca^{2+}, as shown above in Figs. 3 and 4.

Interestingly, the mechanism of $[Ca^{2+}]_i$ oscillations in HSY cells appears to be distinct from what we have previously observed in HSG cells [14]. In HSG cells, removal of external Ca^{2+} abolished CCh-induced $[Ca^{2+}]_i$ oscillations or sustained $[Ca^{2+}]_i$ increase (Fig. 6*A* and 6*B*). Moreover, external Ca^{2+} was required for refill of internal Ca^{2+} stores; we had reported that a second response to CCh was not observed if cells were maintained in a Ca^{2+}-free medium after the first stimulation with CCh [14]. This difference in the pattern of $[Ca^{2+}]_i$ oscillation cannot be accounted for by differences in Ca^{2+} influx. Figure 7 shows that sustained $[Ca^{2+}]_i$ in HSY cells stimulated by either CCh or thapsigargin are higher than those in HSG cells. The initial increase in $[Ca^{2+}]_i$ stimulated by CCh, which mainly represents internal Ca^{2+} release, is not significantly different in the two cell types. The initial increase in $[Ca^{2+}]_i$ stimulated by thapsigargin, which is due to both internal Ca^{2+} release and Ca^{2+} influx, is significantly higher in HSY cells. Thus, although there appears to be a higher level of Ca^{2+} influx in HSY cells, it does not contribute towards the $[Ca^{2+}]_i$ oscillations or refill of the internal Ca^{2+} stores.

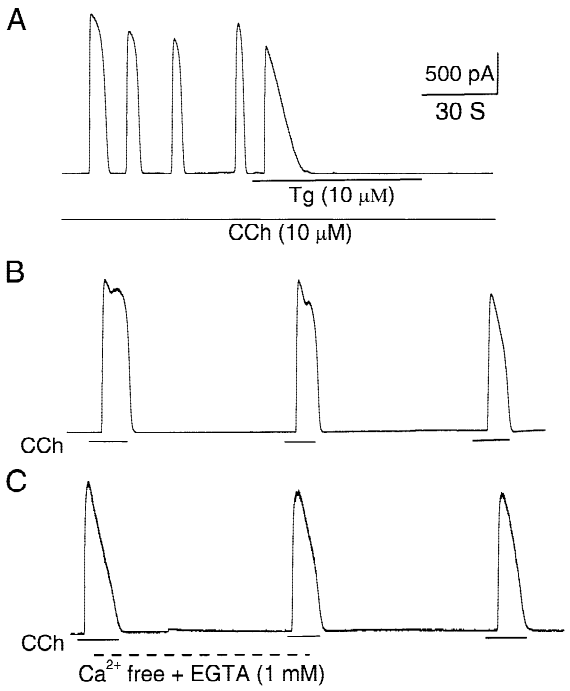

Fig. 5. Role of intracellular Ca^{2+} accumulation in CCh-induced K^+ current in HSY cells. (*A*) The cell was pre-incubated with Ca^{2+}-free medium for 30 sec and maintained in Ca^{2+}-free environment. Tg was applied to the cell during CCh-induced oscillatory K^+ currents as shown by the bar. These are the representative data observed from nine cells. (*B*) The cell was repeatedly stimulated with CCh, for about 30 sec each. (*C*) After the first CCh stimulation, Ca^{2+}-containing medium was replaced with Ca^{2+}-free medium; the duration is shown in the figure by dashed line. Ca^{2+}-containing medium was reintroduced after the second CCh stimulation.

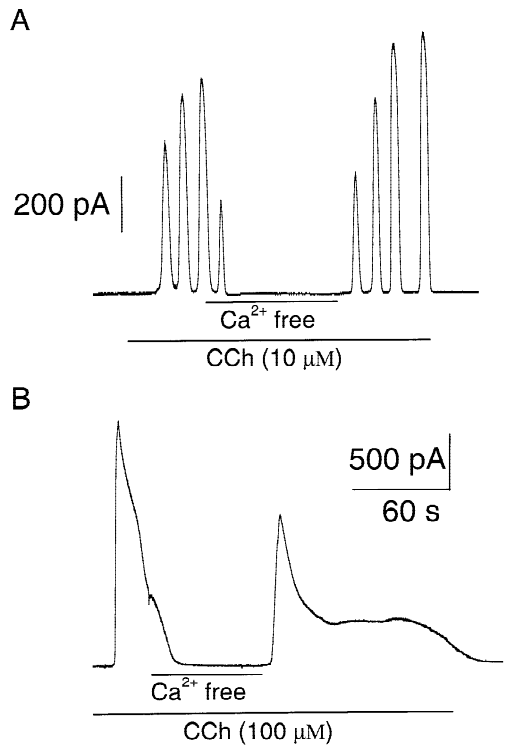

Fig. 6. Effect of external Ca^{2+} on oscillatory and steady state increases in K_{Ca} activity in HSG cells. K^+ current was monitored as described for HSY cells with either 10 μM (*A*) or 100 μM (*B*) CCh. Ca^{2+}-containing perfusion medium was replaced for the duration shown in each trace with Ca^{2+}-free medium. These are representative traces observed from nine cells.

The Status of the Internal Ca^{2+} Store(s) in HSG and HSY Cells

Possible explanations for the observed differences in the pattern of $[Ca^{2+}]_i$ oscillations in HSY and HSG cells are (i) CCh induces less depletion of internal Ca^{2+} stores in HSY cells; (ii) there is more Ca^{2+} in the internal Ca^{2+} stores in HSY cells; or (iii) HSY cells re-accumulate the released Ca^{2+} more efficiently. Thus, the status of the internal Ca^{2+} stores in HSG and HSY cells was further assessed by examining $[Ca^{2+}]_i$ increases stimulated by sequential application of CCh and BHQ to HSG and HSY cells in a Ca^{2+}-free medium (Fig. 8*A*). The increase in $[Ca^{2+}]_i$ induced by 100 μM CCh in the two cells was not significantly different. However, BHQ evoked significantly larger increases in $[Ca^{2+}]_i$ in HSY cells than in HSG cells (Fig. 8*B*, $p < 0.05$, $n = 14$). In aggregate, these data suggest that the internal Ca^{2+} store(s) in HSY cells has a greater capacity to accumulate the Ca^{2+} than that in HSG cells. Alternatively, HSY cells might have a reduced capacity to extrude cytosolic Ca^{2+} due to reduced levels or function of the plasma membrane calcium pump. This would allow more Ca^{2+} to be taken up into the internal stores. The present data do not exclude this possibility and further studies will be required to examine this in greater detail.

The internal Ca^{2+} store was further examined by determining the SERCA and IP_3R content of HSY and HSG cells. HSY cell membranes had a relatively higher level of SERCA than HSG cells (Fig. 9). This is consistent with the suggestion that HSY cells have a higher Ca^{2+}-sequestering ability than HSG cells. Notably, the levels of IP_3R1, IP_3R2, and IP_3R3 were also relatively higher in HSY cells. This indicates that the overall Ca^{2+}-recycling ability of the HSY cell in endoplasmic reticulum is higher than that of HSG cells.

Discussion

The present study demonstrates that CCh-stimulated $[Ca^{2+}]_i$ oscillations in HSY and HSG cells have distinct requirements for external Ca^{2+}. Sustained $[Ca^{2+}]_i$ oscillations in HSG cells were blocked by addition of La^{3+} [14] or removal of Ca^{2+} from the external medium. Conversely, $[Ca^{2+}]_i$ oscillations in HSY cells did not display

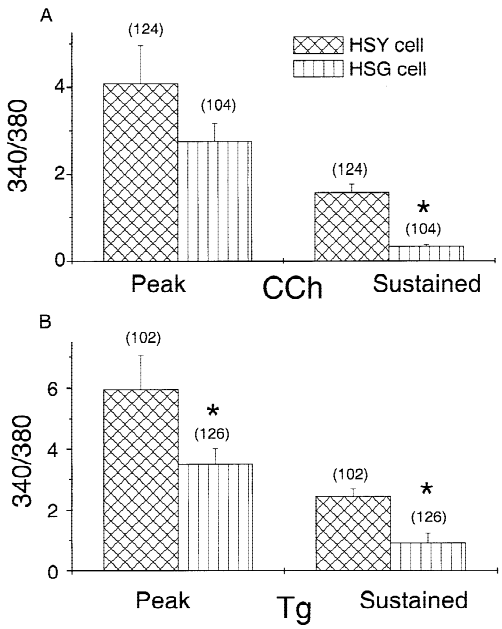

Fig. 7. Carbachol- and thapsigargin-stimulated $[Ca^{2+}]_i$ increases in HSY and HSG cells. Cells were stimulated with 100 μM CCh (*A*) or 2 μM thapsigargin (Tg) (*B*). Initial peak increases and sustained (7.5 minutes after stimulation) increases in $[Ca^{2+}]_i$ (represented by the 340/380 fura-2 fluorescence ratio) were measured. Values marked * indicate values in HSG cells that are significantly different from that in HSY cells ($p < 0.02$; n = number of cells imaged in each case, in a minimum of three separate experiments). Although the peak $[Ca^{2+}]_i$ increase with CCh shows a trend of being higher in HSY cells, this difference is not statistically significant.

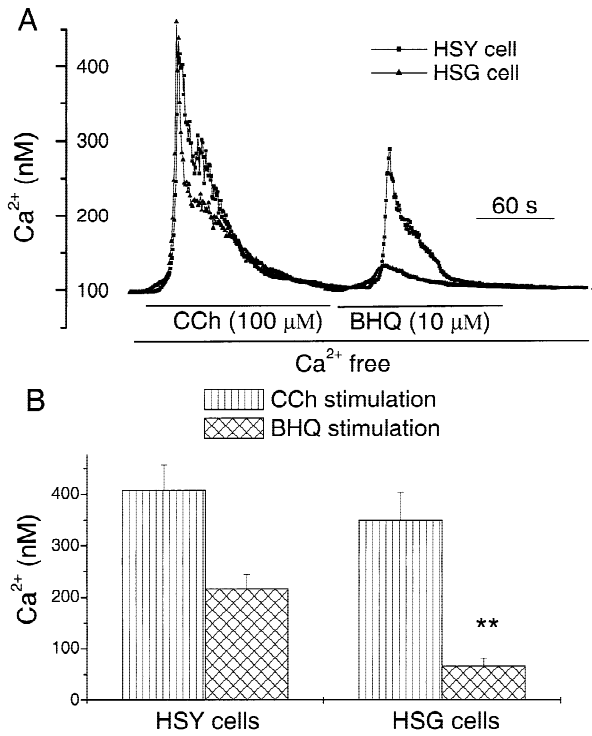

Fig. 8. Ca^{2+} store capacity in HSG and HSY cells. $[Ca^{2+}]_i$ was measured using indo-1 fluorescence in single isolated cells. The cells were maintained in a Ca^{2+}-free medium during the experiment. (*A*) CCh (100 μM) and BHQ (10 μM) were sequentially applied to the cells as indicated. (*B*) Average data obtained from experiments similar to those shown in *A*. Peak increases in $[Ca^{2+}]_i$ induced by CCh in both types of cells are not significantly different. The $[Ca^{2+}]_i$ increase induced by BHQ in HSG cells is significantly lower than that in HSY cells ($p < 0.05$, $n = 14$).

a requirement for external Ca^{2+}. Thus, $[Ca^{2+}]_i$ oscillations in HSG, but not in HSY cells were dependent on Ca^{2+} influx [14]. However, sustained, steady-state $[Ca^{2+}]_i$ increases in both cell types were dependent on the presence of external Ca^{2+}. These data suggest that the mechanisms for $[Ca^{2+}]_i$ oscillations in HSG and HSY cells are different. This is an interesting observation since both these cell lines are of human salivary gland ductal origin, albeit from different salivary glands. Furthermore, both cells have similar types of maxi-K^+ channels (i.e., sensitive to Ca^{2+} and ChTx) and intracellular Ca^{2+} stores (i.e., sensitive to IP_3 and thapsigargin (or BHQ) but not to ryanodine or caffeine). Thus, variations in the type of Ca^{2+} store, or accompanying changes in membrane potential, cannot account for the differential effects of external Ca^{2+} on $[Ca^{2+}]_i$ oscillations in the two cell types.

Many nonexcitable cells show oscillatory changes in intracellular $[Ca^{2+}]$ upon agonist stimulation [2–4, 18–21]. Two main models have been proposed to describe the mechanism of $[Ca^{2+}]_i$ oscillations [2, 6, 7, 23]. The first model proposes that fluctuations in the IP_3 concentration control the oscillations in $[Ca^{2+}]_i$. According to this, an upstream signaling component involved in IP_3 generation is activated in an oscillatory mode, resulting in sequential activation and inactivation of the IP_3R and oscillatory increases in $[Ca^{2+}]_i$. The second model proposes that the concentration of IP_3 does not fluctuate but that the oscillations are determined by the regulation of the IP_3 receptor. It has been reported that low cytosolic and high intraluminal $[Ca^{2+}]$ facilitate Ca^{2+} release by IP_3. In contrast, relatively high cytosolic but low intraluminal $[Ca^{2+}]$ prevent further Ca^{2+} release from the Ca^{2+} store. Thus, it has been suggested that during each oscillation, IP_3R is activated resulting in the release of Ca^{2+} from the store into the cytosol (upstroke). The resulting increase in $[Ca^{2+}]_i$ causes feedback-inactivation of IP_3R, following which $[Ca^{2+}]_i$ is decreased due to resequestration of Ca^{2+} into the store (downstroke). In some cells, $[Ca^{2+}]_i$ oscillations are completely dependent on the presence of external Ca^{2+}. This type of oscillation also appears to be triggered by the activation and inactivation of the IP_3 but in this case refilling of the internal Ca^{2+} store is dependent on external Ca^{2+} and the store-operated Ca^{2+} influx pathway [14]. Thus, during the upstroke, there is store depletion and activation of Ca^{2+}

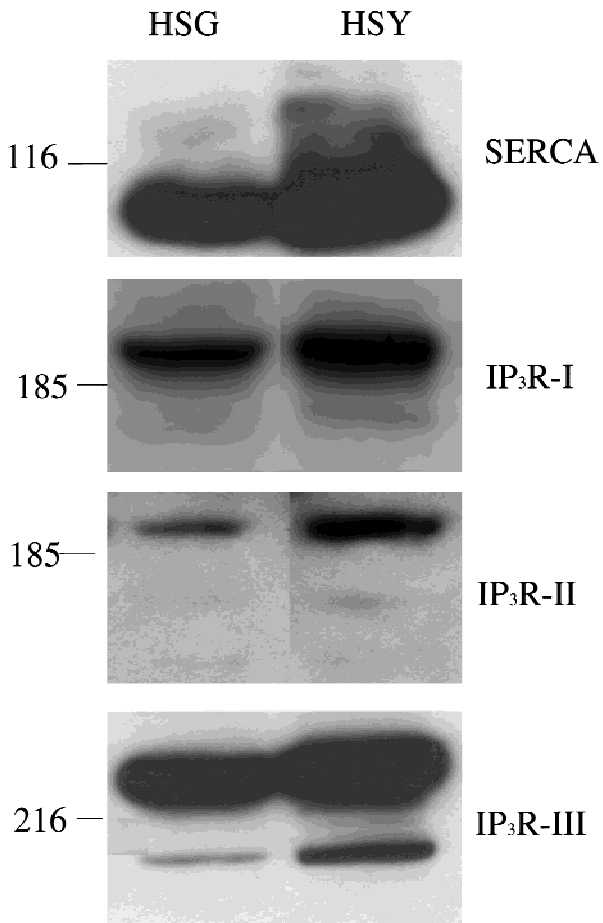

Fig. 9. Relative levels of SERCA and IP_3Rs in HSY and HSG cells. Crude membranes were prepared from HSG and HSY cells. Equal concentrations of protein were loaded on the gel (lane *1:* HSG; lane *2:* HSY). After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Blots were treated with the respective antibodies for SERCA, IP_3R-1, IP_3R-2, and IP_3R-3 followed by the appropriate secondary antibodies, and the proteins were visualized by ECL-reaction. The data shown are representative of three separate experiments.

influx while during the downstroke, when the IP_3 receptor is inactivated, there is refill of internal Ca^{2+} stores and gradual inactivation of store-operated Ca^{2+} influx. Our previous study with HSG cells demonstrated that (i) both oscillatory and sustained increases in the K_{Ca} current in HSG cells were dependent on external Ca^{2+}, and (ii) the K^+ current was not activated when Ca^{2+} influx occurred after the IP_3 receptor had been inactivated (i.e., during refill of the internal Ca^{2+} stores). Thus, we had proposed that Ca^{2+} entering the cell was routed to the region of the K_{Ca} channels via uptake into the store and IP_3-mediated Ca^{2+} release. Our data with HSG cells predict that during each oscillation of $[Ca^{2+}]_i$ the internal Ca^{2+} store is emptied, which stimulates Ca^{2+} influx, and refilling of the store is accomplished by sequestration of the Ca^{2+} entering the cell from the external medium via the store-operated Ca^{2+} entry pathway. A recent study with pancreatic acinar cell is consistent with our data [17].

The model used to explain the $[Ca^{2+}]_i$ oscillations in HSG cells cannot readily explain the nature of the $[Ca^{2+}]_i$ and K_{Ca} current oscillations in HSY cells described above. We have shown that muscarinic receptor stimulation, or intracellular IP_3, induces sustained oscillations in $[Ca^{2+}]_i$ in HSY cells that occur independent of Ca^{2+} influx. These can be distinguished from the sustained, steady-state, increases in $[Ca^{2+}]_i$ which are dependent on Ca^{2+} influx. This is in contrast to the results obtained with HSG cells, where both the sustained oscillations and the steady-state increase in $[Ca^{2+}]_i$ were dependent on external Ca^{2+} and store-operated Ca^{2+} influx [14]. The difference in the extracellular Ca^{2+} requirement of $[Ca^{2+}]_i$ oscillations in HSY and HSG cells is probably not due to a difference in the levels of IP_3 generated by CCh since the magnitude of $[Ca^{2+}]_i$ increase due to internal Ca^{2+} release was similar in both cell types. Further, HSY cells perfused with high $[IP_3]$ also showed a similar effect of external Ca^{2+} removal, i.e., the sustained, steady-state increases in K_{Ca} current and $[Ca^{2+}]_i$ were converted to an oscillatory increase (*data not shown*). Additionally, HSY cells displayed an apparently higher level of Ca^{2+} influx following stimulation by either CCh or thapsigargin. This indicates that the oscillatory component of the $[Ca^{2+}]_i$ increase in HSY cells appears to be independent of the level of IP_3 or the Ca^{2+} influx component. Consistent with the present data, Tanimura and Turner [25] previously reported that 1 μM IP_3 induced oscillations in the $[Ca^{2+}]$ of the internal Ca^{2+} store in permeabilized HSY cells. These oscillations were complemented by oscillations of the $[Ca^{2+}]$ on the external surface, i.e., cytosolic side, of the store, suggesting that during each oscillation, Ca^{2+} is released from and then sequestered back into the internal store. Further, when cytosolic $[Ca^{2+}]$ was buffered, the frequency of these oscillations was decreased. Thus, these investigators concluded intraluminal Ca^{2+} oscillations were not generated due to changes in the IP_3 concentration but most likely due to the biphasic effect of Ca^{2+} on the sensitivity of the IP_3 receptor. They also inferred from these data that $[Ca^{2+}]_i$ oscillations in HSY cells could not be explained by a model involving sequential depletion of the Ca^{2+} stores and Ca^{2+} influx-dependent refilling.

The data presented above demonstrate that HSY cells are not affected by external Ca^{2+} influx during sustained $[Ca^{2+}]_i$ oscillations induced by either CCh or IP_3. Furthermore, we have shown that when HSY cells are stimulated with 100 μM CCh, the stores do not appear to be fully depleted since a second exposure to 100 μM CCh induces a normal $[Ca^{2+}]_i$ increase. Note that the cells were in a Ca^{2+}-free medium and thus there was no Ca^{2+} influx-dependent refilling of internal Ca^{2+} stores prior to this response. Notably, under the same experimental

conditions, HSG cells did not exhibit a response to the second CCh addition [14], suggesting that the Ca^{2+} stores in these cells were depleted under these conditions. Further, we have shown that following CCh stimulation in a Ca^{2+}-free medium, BHQ evoked an additional increase in $[Ca^{2+}]_i$ that was significantly larger in HSY cells than in HSG cells. Based on these data, it can be suggested that (i) the capacity of the internal Ca^{2+} store is larger in HSY cells than HSG cells, or (ii) the Ca^{2+} from the cytosol is recycled back into the ER more efficiently in HSY cells than in HSG cells. While our data do not unequivocally rule out the possibility that HSY cells have a larger Ca^{2+} store, we have clearly demonstrated that HSY cells have relatively higher levels of SERCA and IP_3Rs than HSG cells. Thus, it is most likely that HSY cells recycle the internal Ca^{2+} store; both release as well sequester Ca^{2+} more efficiently than HSG cells. We suggest that in HSY cells, during the downstroke of each $[Ca^{2+}]_i$ oscillation, Ca^{2+} is rapidly taken up from the cytosol into the internal Ca^{2+} store. This, most likely, also leads to a faster inactivation of the store-dependent Ca^{2+} influx pathway. More importantly, bulk of the Ca^{2+} taken up into the store(s) in HSY cells is by resequestration from the cytosol rather than from the external medium. Thus, differences in the capacity of the internal Ca^{2+} store(s) to recycle Ca^{2+} can account for the apparent differences in the mechanism of $[Ca^{2+}]_i$ oscillations in HSY and HSG cells.

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