SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigating the role of intracellular calcium concentration in parotis and skeletal muscle

by

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Introduction

The changes in intracellular calcium concentration ($[Ca^{2+}]_i$) play fundamental and general regulatory roles in controlling cellular homeostasis practically in every organ and tissue. This prominent regulatory role of Ca^{2+} may be attributed to several factors. Firstly, it binds easily to organic molecules due to its bivalent charge and, secondly, for the same reason, it is difficult for it to cross the cell membrane without transport proteins (ion channels or mobile carriers). Thus, cells can maintain low levels of $[Ca^{2+}]_i$ relatively efficiently, which is a prerequisite for the sudden increase in $[Ca^{2+}]_i$, the key signalling step that occurs as a result of a stimulus.

During my doctoral studies, I have investigated two well-defined areas in relation to the regulatory role of changes in $[Ca^{2+}]_i$. In one series of experiments, I investigated different types of agonists acting on the Ca^{2+} channels of the sarcoplasmic reticulum (SR) in skeletal muscle (ryanodine receptors, RyR1), and compared their effects in terms of their efficacy and selectivity. In the second part of my work, I analysed Ca^{2+}-dependent aspects of the regulation of saliva secretion, which resulted in a new, refined model of saliva secretion that describes the secretion process more accurately than before.

Aims

During our experiments carried out on skeletal muscle fibres, we performed a quantitative comparison between three chlorocresol compounds on the RyR1 channel and the SERCA pump in order to find the most selective agonist acting on RyR1. To this end, ionic currents were measured on RyR1 receptors embedded in lipid bilayers; Ca^{2+} transport experiments were performed on heavy and light membrane vesicles isolated from SR; and finally, *ex vivo* skeletal muscle experiments were also performed. The measurements carried out on the acinar cells of salivary glands are based on the results of pilot experiments, which led us to assume that the currently generally accepted mechanism of saliva secretion needs to be refined. This was the aim of our electrophysiological and morphological experiments performed on acinar cells.

Literature review

The ryanodine receptor

The ryanodine receptor (RyR) is a ligand-gated calcium channel in the sarcoplasmic reticulum. The function of RyRs can be modified by a number of endogenous factors (e.g. Ca^{2+}, Mg^{2+}, nucleotides) and exogenous factors (such as methylxanthines or phenol derivatives; in particular, cresols).

Ca^{2+} is the most important natural regulator of RyR1; the other ligands of RyR1 are either unable to activate the channel in the absence of Ca^{2+} or require Ca^{2+} to elicit maximum effect. There is a bell-shaped correlation between the open probability (P_o) of the channel and Ca^{2+} concentration. At nanomolar Ca^{2+} concentrations, P_o is close to zero. Cytosolic Ca^{2+} concentrations up to 50–100 μM activate RyR1, whereas higher Ca^{2+} concentrations inhibit the channel (IC_{50} = 300–500 μM) (Smith et al., 1986; Sárközi et al., 2000; Szigeti et al., 2007). The effect of Ca^{2+} can be explained by an activating and a low-affinity inhibitory binding site located on the cytoplasmic side of the protein.

Mg^{2+} at micromolar concentrations inhibits RyR function. The inhibitory effect of Mg^{2+} is attributable to the fact that Mg^{2+} closes the channel by binding to the Ca^{2+}-binding sites (Smith et al., 1986; Jóna et al., 2001).

Adenine nucleotides activate the RyR channel. ATP has been shown to be the most efficient activator, which in terms of efficiency is followed by ADP, AMP, and cAMP. Simultaneous presence of Ca^{2+} and ATP is required for maximal activation of the channel (Meissner, 1994; Zucchi and Ronca-Testoni 1997).

Among the methylxanthines, caffeine increases the open probability of RyR1 and therefore triggers muscle contractility. It achieves its P_o-boosting effect by increasing the sensitivity of Ca^{2+} activation sites. This is indicated by the fact that caffeine applied at 0.5–2 mM triggers its activating effect at Ca^{2+} concentrations in the μM range, whereas when applied at 5–10 mM, it keeps RyR1 open even at picomolar Ca^{2+} concentrations. The other methylxanthines have similar effects to caffeine. The order of potency of methylxanthines is as follows: methylxanthine > theobromine ~ theophylline > caffeine ~ dimethylxanthine (Zucchi and Ronca-Testoni, 1997). Under *in vitro* conditions, caffeine is the most widely used RyR1 agonist, which has provided invaluable information on the role of RyR1 in muscle function (Endo, 1975; Fryer and

Neering, 1989; Allen and Westerblad, 1995; Hermann-Frank et al, 1999). However, caffeine is far from an ideal experimental tool for studying the regulation of muscle function, partly because the highly hydrophobic molecule is difficult to wash out, and it only induces Ca^{2+} release at high (several mM) concentrations (Westerblad et al, 1998; Wendt and Stephenson, 1983). Moreover, its selectivity is not sufficient, as it has additional effects: it increases cAMP levels in the fibre owing to its inhibitory effect on phosphodiesterases; increases the Ca^{2+} sensitivity of myofibrils; and alters the function of SERCA, IP_3 receptors, and several ion channels (Hirose et al., 1993; Zahradník and Palade, 1993; Islam et al., 1995).

Phenol derivatives play an important role in medicine, the pharmaceutical and cosmetic industry, and dyes. Most natural phenol derivatives such as thymol and its structural analogues (carvacrol, vanillin, cineol) are used for their antifungal and antibacterial activity. Thymol is also used in anaesthesiology as a preservative and stabilising compound of halothane. In the cosmetic and pharmaceutical industry, eugenol and anise aldehyde are also used for their pleasant fragrance. Thymol at submillimolar concentrations induces Ca^{2+} release from SR vesicles isolated from skeletal muscle. Sárközi and colleagues studied the effects of natural phenol derivatives on the skeletal muscle SERCA pump and RyR1; they showed that thymol and carvacrol were the most potent SERCA pump inhibitors, while inducing Ca^{2+} release from heavy SR vesicles of skeletal muscle via RyR1 activation in a concentration-dependent manner. Thymol and carvacrol increase the open probability of the Ca^{2+} channel. Due to its excellent solubility, the reproducibility of the experiments, and its low effective concentration (Kd=160 μM), thymol may even be an ideal RyR1 agonist (Sárközi et al., 2007).

Cresols are phenol derivatives with a methyl group and a hydroxyl group attached to the benzene ring. Depending on the position of these functional groups in relation to each other on the benzene ring, three isomers can be distinguished: ortho-cresol, meta-cresol and para-cresol. The chlorinated versions of these compounds are termed chlorocresols.

The technical difficulties caused by the use of caffeine can be significantly mitigated by the application of chlorocresoles, a group of more specific RyR1 agonists than caffeine itself. The chlorocresol molecule contains a hydroxyl group substituted in a benzene ring, a methyl group, and a chlorine atom. Based on their position relative to each other,

there are three different compounds: 4-chloro-ortho-cresol (4-COC), 4-chloro-meta-cresol (4-CMC), and 3-chloro-para-cresol (3-CPC).
Previous studies have shown that 4-CMC, which is used as a preservative in drugs, increases the binding of tritium-labelled ryanodine to heavy SR vesicles, triggers Ca^{2+} release, increases the open probability of RyR1 channels, and effectively inhibits the SR Ca^{2+} ATPase (Zorzato et al., 1993; Tegazzin et al., 1996; Herrmann-Frank et al., 1996). The selectivity of 3-CPC and 4-COC on RyR1 and the SERCA pump has so far remained unclear, although data is available regarding 4-CMC inhibiting SERCA function (Al-Mousa and Michelangeli, 2009). The different molecular structures suggest that chlorocresols with different structures may stimulate RyR1 and inhibit SERCA to a different extent, and that there may also be differences in the selectivity of the compounds. The aim of our experiments was to explore these differences, which would allow the selection of the most effective and selective RyR1 agonist.

Function of the SR Ca^{2+} pump

Ca^{2+} released into the cytosol is returned to the SR by an active transport mechanism. One of the key steps in SERCA function is phosphorylation of the enzyme, leading to a transient E_1-E_2 conformational change. Along with ATP hydrolysis, the protein in the E_1 conformation releases two Ca^{2+} into the longitudinal tubule of the SR. Subsequently, the Ca^{2+}-bound protein hydrolyses ATP in the presence of Mg^{2+}, during which the pump is phosphorylated, a process that stabilizes its E_2 conformation. SERCA belongs to the group of high affinity Ca^{2+} transport systems (K_m approx. 400 nM), and therefore the pump activity is Ca^{2+}-dependent, with a concentration optimum of around 2 μM Ca^{2+}.

Structure of the cells in the parotid acinus

The cells in the pyramid-shaped serous acini of the parotid gland are structurally and functionally polarized. Their product is the primary saliva.
As a result of this polarisation, the membrane of the acinar cells can also be divided into two parts. The apical (luminal) membrane is the part of the cell with a smaller surface area facing the lumen, where the vesicles filled with the secretory product are located. Fluid and enzyme secretion takes place through the luminal membrane towards the lumen. The relatively broader part of the cell facing the basal lamina is the basolateral membrane. The extent of the apical membrane has been previously estimated to be a relatively small fraction of the total membrane surface. Acinar cells

with a polarized structure are also functionally polarized, which means that the location of transporters and ion channels is also different between the basolateral and apical membranes. The acinar cells of salivary glands produce primary saliva, which is an isosmotic fluid. Fluid and enzyme secretion occurs through the apical membrane. Zonula occludens (ZO), also known as tight junction (TJ), provides the mechanical connection between the acinar cells. The TJ is located close to the apical membrane, in a belt-like manner. In addition to providing cell-cell contacts, the TJ also plays a role in preventing lateral diffusion of membrane proteins, maintaining the polarized structure of acinar cells (Tsukita et al., 2001; Baum, 1993).

The acinar cells are innervated by parasympathetic and sympathetic postganglionic fibres. The reflex mechanism releases acetylcholine (ACh) from the nerve endings of the parasympathetic postganglionic fibres. ACh binds to M_3-type ACh receptors located in the basolateral membrane of the acinar cells (Melvin et al., 2005; Cook et al., 1994). Receptor activation eventually results in the production of IP_3, a secondary messenger, which binds to the IP_3 receptor on the endoplasmic reticulum (ER) and initiates Ca^{2+} release (Clapham, 1995; Ambudkar, 2000; Bootman, 2001). Ion movements are triggered by increased intracellular Ca^{2+} concentration due to parasympathetic stimulation of the acinar cells, which opens Ca^{2+}-dependent Cl^- channels located in the luminal region (Bootman, 2001; Iwatsuki et al, 1985; Maruyama et al., 1986; Maruyama et al., 1983; Petersen, 1992; Petersen and Gallacher, 1988). These channels have been identified as TMEM16A (Yang et al., 2008; Romanenko et al., 2010).

According to the current model of saliva secretion, the process is driven by the Na^+/K+-pump located in the basolateral membrane of acinar cells, and the Na^+ gradient that it generates, which is clearly demonstrated by the fact that saliva secretion can be completely suspended by ouabain (Petersen and Poulsen, 1967; Poulsen, 1974; Silva et al., 1977). Coupled to the Na^+ gradient, the $Na^+/K^+/2Cl^-$ electroneutral cotransporter delivers Cl^- across the basolateral membrane into the intracellular space (Evans et al., 2000; Martinez et al., 1983; Novak et al., 1986). An additional basolateral chloride uptake pathway has been identified, involving the coordinated action of two antiporters, the Na^+/H^+ exchanger (NHE1) and the Cl^-/HCO_3^- exchanger (AE2), but its significance is still controversial (Catalán et al., 1999; Nakamoto et al., 2007). According to the model, only the intracellular Cl^- concentration is increased, because Na^+ and K^+ taken up by the $Na^+/K^+/2Cl^-$ transporter are cycling across the basolateral membrane. The

incoming Na^+ is removed by the Na^+/K^+-pump, while K^+ is cleared through K^+ channels – both across the basolateral membrane. Cl^- flows into the acinus lumen via Ca^{2+}-dependent Cl^- channels in the luminal membrane along the electrochemical gradient, making the acinar cell become electronegative, and therefore creates an electrical gradient for Na^+ (Kasai et al., 1998). During the formation of the primary secretion, Na^+ enters the lumen from the interstitium through a paracellular pathway. Cl^- and Na^+ entering the acinar lumen in this way induce paracellular water influx, making the primary saliva isosmotic (Begenisich and Melvin, 1998). An essential feature of the model is that K^+ entering the cell by different mechanisms leaves the cell via conductive K^+ channels on the same side as the pump, i.e. the basolateral surface.

During the process, the electrochemical driving force for Cl^- is constant, as the counter current of Ca^{2+}-dependent K^+ channels (K_{Ca2+}) continuously provides the negative membrane potential. K^+ can return to the interstitium via at least two types of K^+ channels: the high conductance K_{Ca2+} (BK, KCa1.1) and the medium conductance SK4 channel ($K_{Ca3.1}$). SK4 is a Ca^{2+}-dependent K^+ channel, while BK is regulated in both Ca^{2+} and voltage-dependent manners (Nehrke et al, 2003; Romanenko et al., 2007). Thus, the role of Ca^{2+}-dependent K^+ channels during saliva secretion is to provide a constant electrochemical driving force for Cl^-, so that the counter current K_{Ca2+} constantly maintains a negative membrane potential. K^+ is pumped back into the acinar cell by the Na^+/K^+ ATPase. The important role of K_{Ca2+} is indicated by the fact that fluid secretion can be inhibited by the K_{Ca2+} channel inhibitor paxillin, as well as ouabain. As described above, the Ca^{2+}-dependent secretory regulation relies on the fact that an increase in intracellular Ca^{2+} concentration opens both Cl^- and K^+ channels required for continuous secretion. Previous research has shown that K_{Ca2+} channels are located in the basolateral membrane of cells. The current saliva secretion model is using this to explain that primary saliva contains low levels of K^+, and places the Na^+/K^+ ATPase in the basolateral membrane, the same location as the K_{Ca2+} channel (Catalán et al, 2009; Mangos et al, 1973; Almassy et al, 2012). The results presented in my thesis challenge this classical model of saliva secretion by demonstrating the functional presence of a significant amount of K_{Ca2+} channels and Na^+/K^+-pumps in the luminal membrane. Based on our results obtained from the morphological and functional analyses of transport proteins, we have constructed a new saliva secretion model.

Methods

Methods for studying Ca^{2+} transporters in skeletal muscle

SR microsome isolation from rabbit skeletal muscle

The microsomal fraction containing the SR terminal cisternae (heavy SR vesicles, HSRV) and longitudinal tubule SR vesicles (light SR vesicles, LSRV) were isolated from rabbit skeletal muscle by differential centrifugation and sucrose gradient centrifugation (Sárközi et al., 2007). The LSRV contains the SERCA pump, whereas the HSRV contains both RyR and SERCA pumps.

Calcium flux measurement from SR vesicles

The heavy SR vesicles were suspended in buffer (92.5 mM KCl, 18.5 mM MOPS, 1 mM $MgCl_2$, 1 mM ATP, and 250 μM antipyrylaso III; pH=7.0) in a glass cuvette (Rousseau et al., 1987). The extravesicular Ca^{2+} concentration of the solution was monitored by the transmittance of the metallochromic Ca^{2+} indicator dye antipyrylaso III at 710 nm using a Spex-Fluoromax spectrofluorimeter. At the beginning of the experiment, heavy SR vesicles were loaded with Ca^{2+} using the SERCA pump. After Ca^{2+} uptake was complete, Ca^{2+} release was induced with different doses of chlorocresols. Data were evaluated by determining the amount of Ca^{2+} released, and the rate of Ca^{2+} release.

Ionic current recordings on RyR1 channels

Channel activity was monitored on RyRs fused into an artificial planar lipid bilayer by recording the ionic currents. RyR was incorporated by fusion of heavy SR vesicles into a planar lipid membrane (Sárközi et al., 2007). The lipid bilayer was formed across a 200-μm diameter hole drilled into a delrin cup (measuring chamber; Warner Instruments Inc.), thus separating two solution compartments. The measuring chamber contained a measuring solution with the following composition: 50 mM $CsCH_3O_3S$, 100 μM K_2H_2EGTA, 150 μM $CaCl_2$, 20 mM HEPES; pH=7.2. The lipid solution contained phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in a ratio of 5:4:1 and was dissolved in *n*-decane at 20 mg/mL. After the successful incorporation of RyR1, the free Ca^{2+} concentration on the *cis* side was reduced from 50 μM to 100 nM by the addition of EGTA, and then chlorocresol was added to the solution

corresponding to the cytoplasmic side of RyR1 at the desired concentration. Ionic currents were detected using an Axopatch-200 amplifier, filtered at 1 kHz using an 8-pole Bessel filter, and digitized at 3 kHz. Current signals were recorded using pCLAMP 6.03 software (Axon Instruments). Channel open probability values were determined using Clampfit 10 software.

ATPase activity measurements

The ATPase activity of LSRV vesicles was determined by a coupled enzyme assay in a medium consisting of 100 mM KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM ATP, 0.42 mM phosphoenolpyruvate, 1 μM A23187 ionophore, 0.2 mM NADH, 7.5 U/mL pyruvate kinase, and 18 U/mL lactate dehydrogenase (pH=7.5, t=37°C). The assay was performed with 1 μM ionized Ca^{2+} to ensure maximum SERCA activity. Total hydrolytic activity was measured as the decrease of optical density of the NADH absorption peak at 340 nm (Sárközi et al., 2007).

Measurement of skeletal muscle contractility

One tendon of the extensor digitorum longus muscle excised from rats was attached to the bottom of the measuring chamber, and the other to the arm of the force transducer in the vertical position. Muscle tone was measured under isometric conditions after setting the preload of the muscle to 10 g. The measuring chamber contained Tyrode's solution, to which 4-COC was added at different concentrations.

Methods for studying parotid acinar cells

Isolation of parotid acinar cells

For electrophysiological measurements, parotid acinar cells were prepared by enzymatic dissociation of parotid tissue (Martinez and Cassity, 1983; Romanenko et al., 2010; Thompson and Begenisich 2006). Parotid glands were rapidly removed from sacrificed mice, finely minced with sharp scissors, and dissociated in 28 μg/mL trypsin in a water bath at 37°C for 8 min with continuous shaking. The tissue was then washed with trypsin inhibitor and further digested for 40 min in 10 mL of a collagenase enzyme mixture containing 0.18 Wünsch units/mL Liberase TL (Roche Diagnostics GmbH). After washing, Liberase digestion was continued for another 20 min. Parotid cells were dissociated using a serological pipette.

Immunocytochemistry

Immediately after isolation, the acinar cell clumps were fixed in ice-cold methanol (as a suspension, for 15 min). The cells were then collected by centrifugation and blocked in PBS (phosphate buffered saline) containing 3% BSA. Cells were then incubated with one of the following antibodies: Na^+-K^+ pump antiserum (Abcam), IP_3R3 antiserum (BD Biosciences), and TMEM16A antiserum (Santa Cruz Biotechnology Inc) at dilutions of 1:250, 1:50, and 1:100, respectively. After washing in PBS, cells were probed with Dylight 488 anti-rabbit, Cy3 anti-mouse goat, and Dylight 488 anti-goat secondary antibodies (ThermoFisher Scientific Inc.) at 1:2000 or 1:1000 dilutions. After repeated washing, cells were examined using a Zeiss 510 Meta (Carl Zeiss Microscopy GmbH) confocal microscope.

Immunohistochemistry

Parotid tissue was prepared for immunostaining according to a standard histochemical protocol. Na^+/K^+ pump antibody (Abcam) was applied overnight at 4°C at a dilution of 1:100. Subsequently, cells were probed with Dylight 488 horse anti-rabbit secondary antibody.

Recording ionic currents on parotid acinar cells

During the experiments carried out using the patch-clamp technique in a whole-cell configuration, ionic currents were recorded using an Axopatch 200 amplifier controlled by a Digidata 1322A A/D card (both from Axon Instruments Inc). Sampling frequency was 50 kHz, and the currents were filtered at 5 kHz using an 8-pole Bessel filter. Data were collected and analysed using pClamp 9 software package (Axon Instruments Inc). K^+ current in acinar cells was measured at +40 mV, and Cl^- current at –20 mV. The pipette was filled with an intracellular solution containing 135 mM K-glutamate, 10 mM HEPES, 10 mM NP-EGTA, 2 mM $CaCl_2$ and 0.25 mM Fluo-4-K (pH=7.2). The composition of the external solution was designed to be suitable for the isolated analysis of Ca^{2+} dependent K^+ and Cl^- currents. Accordingly, for measuring K^+ currents, the external solution contained 135 mM Na-glutamate, 5 mM K-glutamate, 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH=7.2). When measuring the Cl^- current, the K^+ current was blocked with tetraethylammonium in the extracellular solution (containing 140 mM TEA–Cl and 10 mM HEPES; pH=7.2). Currents were recorded

using pCLAMP 6.03 software (Axon Instruments) and were analysed using Clampfit 10 software.

Ca^{2+} release using photolysis

Changes in intracellular Ca^{2+} concentrations were monitored by measuring Fluo-4 fluorescence intensity using a monochromator-based imaging system (Polychrome IV) and a high-speed CCD camera (TILL Photonics GmbH). Cells were illuminated at 488 nm and fluorescence was collected through a 525 nm bandpass filter (Chroma Technology Corp.). Imaging was performed at 40–52 ms intervals with 20 ms exposure time. Changes in fluorescence intensity were normalized to the initial fluorescence as follows: $\Delta F/F_0=(F–F0)/F_0$, where F is the recorded fluorescence, and F_0 is the average fluorescence of the first 10 frames of the image sequence. Histograms were scaled to 4096 levels and pseudo-coloured. Photolysis of NP-EGTA (caged Ca^{2+}) was performed using a flashing UV laser and a custom-designed condenser (Almassy et al., 2012). A 375 nm diode laser (Toptica Photonics AG) was connected to a TE200 microscope (Nikon Corp.) through an optical fibre and a UV condenser (TILL Photonics GmbH.). The laser was focused onto the sample plane using a x40 oil immersion objective (Nikon Corp.). At half maximum intensity of the laser spot, the laser spot coverage was approximately 0.7 μM in the x and y planes, and 2.0 μM in the z plane, a resolution sufficient to illuminate a predefined small area of the acinar cell in isolation. This setup allowed us to trigger photolysis in isolation in the apical and basolateral regions, i.e. to induce localized Ca^{2+} release. Cells were illuminated for 40–52 ms, with laser power controlled by software between 4 and 6 mW. Current recordings, fluorescence image acquisition, and laser exposure triggering were synchronized using a Polychrome IV driver and controlled by Vision software package (TILL Photonics GmbH.).

Results

Results on skeletal muscle

Effects of caffeine on Ca^{2+} release in HSR vesicles

The effect of caffeine and chlorocresols on Ca^{2+} release from SR was studied on HSR vesicles obtained from microsome fractions. HSR vesicles were suspended in buffer containing the Ca^{2+} indicator APIII, which was used to monitor the Ca^{2+} concentration of the extravesicular space. At the beginning of each experiment, the vesicles were loaded with equal amounts of Ca^{2+} using the Ca^{2+} pump. Following the addition of caffeine, the relative transmittance of the extravesicular space decreased significantly when the applied caffeine concentration reached 4 mM, indicating that caffeine triggered Ca^{2+} release from Ca^{2+}-loaded vesicles. The rate of caffeine-induced Ca^{2+} release was the same in case of both 4 and 8 mM of caffeine, but slightly lower for 16 mM of caffeine. These results also clearly show that experimental work with caffeine is difficult to standardize, its use is pharmacologically disadvantageous, as its action tends to follow an all-or-nothing law, which raises the need for an agonist with a more specific and concentration-dependent effect on RyR. Therefore, during the rest of this work, I have tested the effects of such candidate compounds (chlorocrestols), which appear to be more suitable on RyR1 and the SERCA pump.

Effect of chlorocresols on Ca^{2+} release in HSR vesicles

The effect of three chlorocresol molecules (4-COC, 4-CMC and 3-CPC) on Ca^{2+} release was studied under similar experimental conditions. To this end, different concentrations of chlorocresols (100, 300, and 500 μM) were injected into the cuvette, followed by a rapid decrease in transmittance of the extravesicular medium, indicating that chlorocresols triggered Ca^{2+} release from HSR vesicles. To verify the specificity of the reaction, 500 μM of chlorocresol was applied in the presence of the RyR1 inhibitor ruthenium red (RR, 5 μM). In these cases, Ca^{2+} release was not detected, i.e. ruthenium red pre-treatment completely blocked the effect of chlorocresol, indicating the specificity of the reaction.

The rate of Ca^{2+} release at different chlorocresol concentrations was determined by fitting a line to the initial phase of the intensity change. The reciprocal values of the slope of the line were plotted against chlorocresol concentration values. The data were

fitted with the Hill equation to determine the EC_{50} values, which were 175±39 μM for 4-CMC, 182±37 μM for 3-CPC, and 113±36 μM for 4-COC.

The relative amount of Ca^{2+} released was also determined at different chlorocresol concentrations. The half-effective concentrations were 121±20 μM for 4-CMC, 71±7 μM for 3-CPC, and 55±14 μM for 4-COC.

The total amount of Ca^{2+} released in the presence of the highest (500 μM) concentration was different for the different chlorocresols. Relative steady-state transmittances showed that 3-CPC released twice as much Ca^{2+} as 4-CMC. This parameter was also slightly higher for 4-COC, but the difference was not statistically significant.

Effect of chlorocresols on SERCA ATPase activity

The effect of different chlorocresols on SR Ca^{2+} pumps was assessed by measuring the ATPase activity of LSR vesicles in a concentration-dependent manner. 4-CMC and 4-COC clearly inhibited ATPase activity. Fitting a Hill equation to the data yielded the following IC_{50} values: 167±8 μM for 4-CMC and 1370±88 μM for 4-COC. 3-CPC showed a biphasic effect: at lower concentrations, it stimulated the pump (EC_{50}=91±17 μM), whereas at higher concentrations, it had an inhibitory effect (IC_{50}=848±90 μM) on the ATPase activity of SERCA.

Effect of 4-COC on ionic currents of single RyR1 channels

To gain more information on the molecular mechanism of the effect of 4-COC, the compound was further tested on single RyR1 channel currents embedded in a lipid bilayer. Under control conditions, RyR1 currents were recorded in the presence of 100 nM Ca^{2+} on the cytoplasmic side of the channel. In this condition, the channels are closed most of the time and therefore have a low open probability (average of three experiments; P_o=0.006). When the channels were treated with 110 μM 4-COC, the open probability increased fourfold (P_o=0.025), due to more frequent opening events.

Muscle contracture triggered by 4-COC

In the next series of experiments, we tested whether 4-COC modified the mechanical activity of skeletal muscle. The tone of extensor digitorum longus muscle excised from rats was continuously monitored using a force transducer while the muscle was treated with cumulatively increasing concentrations of 4-COC. First, 1.6 mM 4-COC

concentration was set, which proved to be ineffective. Then, by administering additional 4-COC injections, we increased the concentration by 0.2 mM increments up to 2 mM, which was able to slightly enhance muscle tone. When the concentration of 4-COC reached 2.2 mM, there was a sudden marked increase in muscle tone. DMSO alone (used as a vehicle) was ineffective. These results suggest that 4-COC induces skeletal muscle contracture through specific activation of the Ca^{2+}-releasing apparatus.

Results obtained on parotid cells

Apico-basal localisation of Ca^{2+}-dependent K^+ and Cl^- channels

Although previous research has shown that K_{Ca2+} channels were clearly located in the basolateral membrane of cells, recent mathematical models have challenged this view (Palk et al., 2010). To clarify this issue, whole-cell patch-clamp measurements were performed to generate spatially localised Ca^{2+} release in the area below the basal or apical membrane of the cell using flashing UV light. In the experiments, Ca^{2+} levels were elevated in the apical or basal region of the acinar cell. The Ca^{2+} concentration measurements showed that the Ca^{2+} signal did not affect the opposite lateral region and remained localised, i.e. no Ca^{2+} transients appeared in the distal region of the cell. In the case of apical Ca^{2+} release, a robust increase in K^+ and Cl^- currents was recorded in parallel with the Ca^{2+} signal, whereas basally applied Ca^{2+} release did not activate either K^+ or Cl^- currents. The Cl^- current serves as a functional marker of the apical membrane, i.e. it indicates that the increase in Ca^{2+} concentration was indeed adjacent to the apical membrane and that it was of sufficient magnitude to activate Ca^{2+}-dependent ionic currents.

These results suggest that Ca^{2+}-dependent K^+ channels are located in the apical membrane of acinar cells – similar to Ca^{2+}-dependent Cl^- channels. This challenges the foundations of the previously known saliva secretion model, as it raises the question of why, despite the significant luminal K^+ leakage, the K^+ concentration in primary parotid saliva is low (~5 mM). A possible answer to this question could be that the apical membrane also contains Na^+/K^+-pump molecules that reabsorb secreted K^+. This possibility was further investigated by employing immunofluorescence.

Apico-basal localisation of the Na^+/K^+-pump

The location of the Na^+/K^+-pump in the membrane of parotid acinar cells was studied by immunofluorescence using anti-Na^+/K^+-pump antibodies. The Na^+/K^+-pump was detectable along the entire circumference of the plasma membrane – including the apical membrane. In these experiments, labelling of the apical region was performed by IP_3 receptor staining. These experiments demonstrate that, contrary to previous hypothesis, the Na^+/K^+-pump is also prominently present in the apical membrane. In contrast to the immunocytochemical results obtained on acinar cells, immunohistochemical studies on ductal cells showed that only the basolateral, rather than the apical membrane, stained for the Na^+/K^+-pump. These results demonstrate the specificity of the antibody, and show that the Na^+/K^+-pump is translocated to the apical membrane only in acinar cells.

Determining the relative size of the apical membrane

Since Cl^- channels are confined to the apical membrane of acinar cells, they can be used as an excellent and specific marker for determining the relative extent of the apical membrane. The presence of a Cl^- channel (which was identified as the TMEM16A (ANO1) protein) in the apical membrane was detected by immunofluorescence. The staining clearly labels the apical membrane. Contrary to previous literature data that the apical membrane accounts for only 5–8% of the total cell membrane, our staining identified a significant portion of the membrane as apical membrane, which we estimate to account for approximately 30% of the total membrane surface.

New saliva secretion model

My results on salivary gland acinar cells do not fully fit into the previously accepted model of the mechanism of saliva production. Indeed, we found that (1) Ca^{2+}-dependent K^+ channels are activated only in the apical region of the salivary acinar cells; (2) the Na^+/K^+-pump is also massively present in the apical membrane in acinar cells; and (3) the apical membrane of acinar cells is significantly larger than previously thought. In view of the above, it is necessary to hypothesize a new model of salivary secretion. This differs from the old model in that Na^+ entry into the lumen is not exclusively via a paracellular pathway, but also via a transcellular, primarily active Na^+ entry mechanism through the Na^+/K^+-pump in the apical membrane. Based on our results, Elias Siguenza and James Sneyd (Department of Mathematics, University of

Auckland, New Zealand) have performed model calculations that describe more accurately than before the events that occur during saliva secretion (Almássy et al., 2018).

Discussion

Analysis of the effects of chlorocresols in skeletal muscle

Our experiments show that of the three chlorocresol stereoisomers tested, 4-COC was the most potent and selective RyR1 agonist, as it had the lowest EC_{50} value for Ca^{2+} release and, more importantly, 4-COC had the weakest influence on SERCA pump activity (i.e. 4-COC had the highest IC_{50} value of 1370 μM).

When comparing the chlorocresols at the maximum applied concentrations (500 μM), 3-CPC had the strongest effect on Ca^{2+} release, but this compound cannot be exploited as a selective agonist due to the complex biphasic SERCA effect. In fact, 3-CPC stimulated SERCA pump function at low doses (EC_{50}=91 μM), whereas it had an inhibitory effect at higher concentrations (IC_{50}=848 μM). Based on the pharmacological properties studied, we suggest the use of 4-COC instead of 4-CMC for RyR1 activation, because it is a more selective and more effective Ca^{2+}-releasing agonist than 4-CMC (and caffeine), which may be advantageous under specific experimental conditions. Although the activity of 4-COC is not qualitatively different from that of 4-CMC, it is a much more selective agonist on the RyR1 receptor. Thus, under experimental conditions, one can presumably expect less interference from changes in SERCA activity when using 4-COC.

Mechanism of salivary secretion in parotid acinar cells

Our experiments conducted on parotid acinar cells clearly demonstrate the presence of K^+ channels in the apical membrane and that the density of K^+ channels is much higher than that in the basolateral membrane. It follows directly that there must be significant K^+ secretion towards the lumen in response to acinar cell stimulation, which is contradicted by the low K^+ content of primary saliva. This contradiction can be resolved if the K^+ exiting the apical side through K^+ channels is returned by some mechanism to the interior of the cell. Our results suggest that this mechanism is the high abundance of Na^+/K^+-pumps in the apical membrane. Indeed, we detected not only Cl^- channels but also Na^+/K^+-pump molecules in the apical membrane using immunocytochemistry. The significance of this is underpinned by the fact that according to our results, the apical membrane surface area is estimated to be about 30% of the total membrane surface area instead of the previously thought low (<8%)

figures, allowing a significant transcellular active Na^+ transport towards the lumen. These results contradict the previously accepted secretion model for parotid acinar cells, which assumes virtually exclusively paracellular Na^+ transport in addition to transcellular Cl^- transport, consistent with the localisation of the Na^+/K^+-pumps in the basolateral membrane.

The available literature, some of which is quite contradictory, has significantly underestimated the apical surface of acinar cells (Bundgaard et al., 1977; Conteas et al., 1986; Garrett et al., 1998; Garrett et al., 1992; Iwano et al., 1987; Nakagaki et al., 1978; Petersen, 1971; Winston et al., 1988; Winston et al., 1990). In light of this, the significance of any Na^+/K^+-pump molecule in the apical membrane did not seem to be a major issue, and the consensus was that their role, if any, was minimal (Garrett et al., 1998; Petersen and Poulsen, 1967). The fundamental error in the definition of the apical membrane surface was based on electron microscopy images where the apical membrane surface was identified by the central holes between cells, ignoring those quite considerable intercellular membrane sections that also belong to the apical membrane (Larina and Thorn, 2005). In contrast, our own immunofluorescence results using TMEM16A suggest that the apical membrane accounts for at least 30% of the total membrane surface area.

Based on our results, we had to modify the previously widely accepted canonical secretion model by incorporating a significant amount of Ca^{2+}-dependent K^+ channels and Na^+/K^+-pumps into the apical part of the plasma membrane, in addition to the TMEM16A Cl^- channels. Since both are activated by Ca^{2+}, the apical colocalization of K^+ and Cl^- channels provides a convenient regulatory mechanism to stimulate saliva secretion. The role of apically localised K^+ channels, on the one hand, is to prevent, or compensate for, the depolarisation due to Cl^- efflux mediated by their outward current, which would prevent further Cl^- efflux. On the other hand, K^+ exiting through the apical membrane is carried back into the cell by the Na^+/K^+-pump, which is also present apically, while this mechanism also contributes to Na^+ secretion into the lumen. Thus, in our refined model, the secondarily active paracellular pathway is complemented by the primarily active transcellular mechanism.

Based on our results, Elias Siguenza and James Sneyd (Department of Mathematics, University of Auckland, New Zealand) performed model calculations using the K^+ and Na^+ concentrations predicted by the old and new models for primary saliva, modelling the magnitude of the current generated by paracellular Na^+ transport, and the intensity

of saliva secretion (Almássy et al., 2018). In all cases, the calculations based on the new model better approximated the measured values than the calculations based on the old model. We can therefore conclude that the new model we propose describes more accurately than before the events during saliva secretion.

Summary

The work presented in my PhD thesis consists of two parts: (1) investigating the effect of various chlorocresol stereoisomers, known agonists of the ryanodine receptor of the skeletal muscle (RyR1), in order to find the most specific RyR1 agonist among them as a caffeine alternative; and (2) investigating the localization of Ca^{2+} dependent ion channels in parotid acinar cells.

Experiments investigating the Ca^{2+}-releasing action of the chlorocresol isomers demonstrated that the most potent isomer was 4-chloro-orto-cresol (4-COC) (EC_{50}=55±14 μM), although 3-chloro-para-cresol (3-CPC) was more effective, as it was able to induce higher magnitude of Ca^{2+} flux from isolated terminal cisterna vesicles. Nevertheless, 3-CPC stimulated the hydrolytic activity of the SERCA Ca^{2+} pump with an EC_{50} of 91±17 μM, while 4-COC affected SERCA only in the millimolar range (IC_{50}=1370±88 μM). The IC_{50} of 4-chloro-meta-cresol (4-CMC) for SERCA pump was 167±8 μM, indicating that 4-CMC is not a specific RyR agonist either, as it activated RyR in a similar concentration (EC_{50}=121±20 μM). Our data suggest that the use of 4-COC might be more beneficial than 4-CMC in such experiments when Ca^{2+} release should be triggered through RyRs without influencing SERCA activity.

In the second part of my project, using a combination of single-cell electrophysiology and Ca^{2+}-imaging, we demonstrated that photolysis of Ca^{2+} close to the apical membrane of parotid acinar cells triggered significant K^+ and Cl^- current, indicating that a substantial amount of K^+ is secreted into the lumen during stimulation of the parotid gland. Nevertheless, the K^+ content of the primary saliva is relatively low, suggesting that K^+ might be reabsorbed through the apical membrane. Therefore, we investigated the localization of Na^+/K^+ pumps in acinar cells. We showed that the pumps appear evenly distributed throughout the whole plasma membrane, including the apical pole of the cell. Based on these results, we created a new model of salivary fluid secretion, where the Na^+/K^+ pump reabsorbs K^+ from and secretes Na^+ to the lumen, which can partially supplement the paracellular Na^+ pathway.

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List of publications related to the dissertation

1. **Skaliczki, M.**, Lukács, B., Magyar, Z. É., Kovács, T., Bárdi, M., Novák, S., Diszházi, G., Sárközi, S., Márton, I., Péli-Szabó, J., Jóna, I., Nánási, P. P., Almássy, J.: 4-chloro-orto-cresol activates ryanodine receptor more selectively and potently than 4-chloro-meta-cresol. *Cell Calcium. 88*, 102213, 2020.
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2. Almássy, J., Siguenza, E., **Skaliczki, M.**, Matesz, K., Sneyd, J., Yule, D. I., Nánási, P. P.: New saliva secretion model based on the expression of Na+-K+ pump and K+ channels in the apical membrane of parotid acinar cells.
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List of other publications

3. Almássy, J., Diszházi, G., **Skaliczki, M.**, Márton, I., Magyar, Z. É., Nánási, P. P., Yule, D. I.: Expression of BK channels and Na+-K+ pumps in the apical membrane of lacrimal acinar cells suggests a new molecular mechanism for primary tear-secretion.
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