Heat acclimation and heat stress have different effects on cholinergic-induced calcium mobilization

PAVEL KASPLER AND MICHAL HOROWITZ
Division of Physiology, Faculty of Dental Medicine, and Department of Physiology, The Hebrew University, Jerusalem 91120, Israel

Received 28 September 2000; accepted in final form 2 February 2001

Kaspler, Pavel, and Michal Horowitz. Heat acclimation and heat stress have different effects on cholinergic-induced calcium mobilization. Am J Physiol Regulatory Integrative Comp Physiol 280: R1688–R1696, 2001.—There is evidence that the signal transduction array responsible for the secretion of water in evaporative cooling by the submaxillary gland of the rat is subject to heat acclimatory responses. The objectives of the present study were 1) to examine whether heat acclimation affects intracellular Ca\(^{2+}\) mobilization and, in turn, submaxillary glandular responsiveness; 2) to assess whether the acclimatory responses differ from those evoked on heat stress (HS). Experiments were conducted on submaxillary glands of rats acclimated at 34°C for 0, 2 [short-term heat acclimation (STHA)], and 30 [long-term heat acclimation (LTHA)] days. The resting cytosolic calcium concentration ([Ca\(^{2+}\)]\(_{s}\)) and the carbamylcholine-evoked calcium signal ([Ca\(^{2+}\)]\(_{i}\)) of dispersed glandular cells were measured using the fluorescent dye fura 2 AM. Inositol-1,4,5-trisphosphate (IP\(_3\))-sensitive endoplasmic reticulum Ca\(^{2+}\) stores were determined in permeabilized cells using fura 2 potassium salt. STHA resulted in a drop in both [Ca\(^{2+}\)]\(_{s}\) and IP\(_3\)-sensitive Ca\(^{2+}\) stores. On LTHA, the [Ca\(^{2+}\)]\(_{i}\) amplitude reverted to the preacclimation value, whereas the IP\(_3\)–sensitive Ca\(^{2+}\) stores remained low. The drop in [Ca\(^{2+}\)]\(_{i}\) on STHA is in accord with the decreased glandular output (measured by \(^{86}\)Rb efflux) observed during this acclimation phase. However, after LTHA the enhanced glandular output despite reduced [Ca\(^{2+}\)]\(_{i}\), levels suggests an increased efficiency of cellular secretory mechanisms in that group. Collectively, the alterations in [Ca\(^{2+}\)]\(_{i}\) support our biphasic acclimation model (Horowitz M, Kaspler P, Marmari Y, and Oron Y. J Appl Physiol 80: 77–85, 1996.). In nonacclimated glands, HS caused an elevation in [Ca\(^{2+}\)]\(_{i}\), coincidently with a decrease in the IP\(_3\) Ca\(^{2+}\) stores. In contrast, [Ca\(^{2+}\)]\(_{i}\), in both STHA and LTHA glands was not affected by HS, despite a marked increase in the IP\(_3\)-sensitive Ca\(^{2+}\) stores in the LTHA glands. The opposing responses to HS and heat acclimation in calcium signaling and stores confirm the specificity of each process.

muscarinic signaling; salivary gland; evaporative cooling; intracellular calcium

HEAT ACCLIMATION INCREASES the sensitivity and efficiency of the evaporative cooling system (26, 32). Namely, less oxygen is consumed per volume of water secreted, and there is less secretion per larger wetted area (32). Furthermore, at a similar stimulation frequency, the heat-acclimated gland produces, over time, a larger secreted volume than that before acclimation. These changes are attributable to celluarily localized adaptations, e.g., membrane receptor density or affinity and hyperplasia and hypertrophy, elicited by temporally varying neurohumoral and intracellular responses during the process of heat acclimation (15, 17).

In the rat salivary gland, as well as in the sweat glands of many other species, the major pathway for water secretion in evaporative cooling is via muscarinic signaling. This pathway has been extensively studied with respect to heat stress and heat acclimation in the submaxillary salivary glands of the acclimating rat model. Horowitz et al. (15) showed that short-term heat acclimation (STHA) upregulates the density of the muscarinic receptors (MR) and lowers their affinity. An altered ratio of low-affinity to high-affinity MR subtype populations was also observed together with changes in the magnitude of the calcium signal evoked by supramaximal concentrations of carbamylcholine (CCh). Under similar conditions, in the rat parotid, Fujinami et al. (7–9) reported an array of transient cellular responses involving the upregulation of MR, coincident with the reduced generation of inositol-1,4,5-trisphosphate (IP\(_3\)), a second messenger in the MR transduction cascade, and a drop in Ca\(^{2+}\) mobilization in response to IP\(_3\) administration. This was followed by a decrease in carbachol-stimulated IP\(_3\) generation and CCh-evoked calcium concentration signal ([Ca\(^{2+}\)]\(_{i}\)) amplitude. In contrast to STHA, long-term heat acclimation (LTHA) has been less studied. We know only that on LTHA, further upregulation of MR density occurs, increasing to preacclimation affinity (13, 15).

Collectively, the temporal biochemical changes, which have been measured in several isolated gland preparations, are time correlated with impaired glandular responsiveness during STHA, followed by the resumption of normal glandular function together with increased sensitivity and responsiveness on LTHA. The evidence accumulated thus far leads us to hypothesize that increased glandular sensitivity is achieved...
through greater receptor affinity, whereas augmented capacity is achieved through increased receptor density or glandular size.

In contrast to heat acclimation, heat stress either has no effect on, or downregulates MR and increases the affinity of the MR, in both salivary glands and in salivary gland cell line, HSY. Stevenson et al. (35) and Calderwood et al. (3) reported elevated IP3 levels and increased calcium influx in response to heat stress in several cell lines, i.e., an opposite response to that deduced for acclimation homeostasis. Collectively, these findings confirm differences between the heat stress effects and the acclimatory response.

In the MR signaling pathway, cytosolic calcium signaling provides an important mediation component triggering many cellular processes. Hence, the aim of the present study was twofold: 1) to examine whether heat acclimation affects intracellular Ca2+ mobilization and, in turn, the responsiveness of the rat submaxillary salivary gland; 2) to assess the specificity of heat acclimation vs. heat stress specificity. For this purpose, the quantity of the calcium present in the endoplasmic reticulum and calcium signals were measured and analyzed with respect to the physiological functioning of the salivary glands of heat-acclimated rats. We also studied the calcium signals during heat stress superimposed on heat acclimation. Our data show that the endoplasmic reticulum IP3 calcium stores and the calcium signals are subject to thermal adaptation.

Cumulatively, our findings indicate that heat acclimation reduces both the evoked calcium signal and the cellular calcium stores, despite an enhanced secretion in the LTHA phase. This may suggest that acclimation leads to an increased efficacy of cellular processes distal to the calcium signal. Heat stress differed from heat acclimation by increasing the evoked calcium signal, suggesting that heat acclimation is a specific thermal response. On acclimation, acclimatory adaptations blunt the heat stress effect.

MATERIALS AND METHODS

Heat acclimation and heat stress. Male rats (Rattus norvegicus, Sabra strain, albino var.; Harlan, Jerusalem) weighing 200–250 g were used. The animals were assigned to normothermic (controls, C) or heat-acclimated (AC) groups. All animals were kept under a 12:12-h light-dark cycle. C rats were kept at 24 ± 1°C, whereas heat acclimation was achieved by continuous exposure to 34 ± 1°C and 30–40% relative humidity, for 2 days (STHA) or 30 days (LTHA) (14). Each group was further subdivided into rats that did not undergo any further treatment and those subjected to heat stress at 41 ± 1°C for 2 h. To assess the effect of the treatments on the rectal temperature of the animals was measured using rectal thermistors (YSI 402; Yellow Springs, OH) inserted 6 cm beyond the anal sphincter (13, 15).

The basal cytosolic calcium level ([Ca2+]i), the [Ca2+]s, and the IP3-sensitive calcium stores were measured in all the experimental groups. Our previously published database characterizing the physiological features of the acclimated submaxillary gland in response to CCh stimulation was used to establish the [Ca2+]i-glandular function correlation. All experiments were performed in accordance with guidelines approved by the Hebrew University Committee for Animal Experimentation.

Dispersed cell preparation. Animals were killed by cervical dislocation. The submaxillary glands from each animal were quickly excised, thoroughly minced, and incubated in 10 ml of digesting medium consisting of Krebs bicarbonate buffer (in mM: 117 NaCl, 4.2 KCl, 1.2 KH2PO4, 1.2 MgCl2, 24 NaHCO3, and 1 g/l glucose, pH 7.4) containing 5.4 U collagenase and 20 μU hyaluronidase and were aerated with a mixture of 95% O2-5% CO2 and shaken for 1 h at 37°C. The suspension was homogenized each 10 min by 10 passages through a Pasteur pipette. The nondigestive debris was discarded, and the suspension was centrifuged (10 g, 1 min) to remove the digesting medium. The pellet was then washed twice and resuspended in Krebs bicarbonate buffer containing 2.5 mM CaCl2 and 1% BSA (fraction V, Sigma). Resting Ca2+ level in dispersed glandular cells. [Ca2+]i was measured for the salivary gland cells of each experimental rat group immediately after termination of the dispersed cell preparation. Likewise, [Ca2+]i was measured in the presence of gradually elevated extracellular concentrations of Ca2+ to get some indications on the capacity of the cell membrane of the various groups to regulate [Ca2+]i at different external calcium loads. Salivary gland cell suspensions were loaded with fura 2 AM (6 μM) at 37°C with continuous aeration and shaking, as before, for 30 min, then washed and resuspended in fresh Krebs containing calcium (2.5 mM) but without BSA. The cells were washed free of fura 2; the pellet was washed twice in calcium-free Krebs, containing 10 mM EGTA, and resuspended in the same medium. Resting [Ca2+]i, was determined by establishing a calcium dose-response curve for resting [Ca2+]i. The external calcium solution was prepared from a 250 mM stock Ca2+ solution added to the experimental cuvette stepwise under constant stirring to establish a dose-response curve within a range of 0–2.5 mM. The [Ca2+]i was measured using a fluorimetric ratio system (PTI). To determine the actual free Ca2+ concentration obtained at each dilution point, free Ca2+ levels were measured before the experiments using the ratiometric fura 2 K salt as the fluorescent dye (see below). [Ca2+]i was measured at each external Ca2+ concentration. The maximal response was calculated by fitting to the dose-response curve.

CCh-evoked Ca2+ signal in dispersed glandular cells. The cell suspension was loaded with fura 2 AM as before. The cell pellet was resuspended in 18 ml of the same Krebs solution and divided into six aliquots (3 ml) to obtain a dose-response curve for the muscarinic agonist CCh (0.5–300 μM). [Ca2+]i was measured as above.

Intracellular Ca2+ store responsiveness in permeabilized cells. The Ca2+ store responsiveness was measured according to Falsafi et al. (6) with some modifications. Cell suspensions from salivary glands were treated as above. The cells in the final pellet were permeabilized by incubation in 25 mg/l saponin for 2.5 min at room temperature as determined in preliminary experiments using trypan blue. The permeabilized cells were washed three times in Krebs buffer and resuspended in 3 ml Krebs containing 180 mM Ca2+, 1 mM (2.5 mM) but without BSA. The cells were washed free of fura 2; the pellet was washed twice in calcium-free Krebs, containing 10 mM EGTA, and resuspended in the same medium. Resting [Ca2+]i, was determined by establishing a calcium dose-response curve for resting [Ca2+]i. The external calcium solution was prepared from a 250 mM stock Ca2+ solution added to the experimental cuvette stepwise under constant stirring to establish a dose-response curve within a range of 0–2.5 mM. The [Ca2+]i was measured using a fluorimetric ratio system (PTI). To determine the actual free Ca2+ concentration obtained at each dilution point, free Ca2+ levels were measured before the experiments using the ratiometric fura 2 K salt as the fluorescent dye (see below). [Ca2+]i was measured at each external Ca2+ concentration. The maximal response was calculated by fitting to the dose-response curve.
Table 1. Resting cytosolic Ca\(^{2+}\) level in dispersed glandular cells

<table>
<thead>
<tr>
<th></th>
<th>Series 1</th>
<th>Series 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>([\text{Ca}^{2+}]_c)</td>
<td>([\text{Ca}^{2+}]_c)</td>
</tr>
<tr>
<td>C</td>
<td>96.9 ± 14.0</td>
<td>130.7 ± 13.7</td>
</tr>
<tr>
<td>STHA</td>
<td>94.5 ± 11.6</td>
<td>141.1 ± 8.1</td>
</tr>
<tr>
<td>LTHA</td>
<td>110.2 ± 23.8</td>
<td>120.5 ± 7.5</td>
</tr>
<tr>
<td>C-HS</td>
<td>78.6 ± 5.0</td>
<td>141.1 ± 18.3</td>
</tr>
<tr>
<td>STHA-HS</td>
<td>93.5 ± 6.1</td>
<td>137.1 ± 12.2</td>
</tr>
<tr>
<td>LTHA-HS</td>
<td>77.1 ± 7.8</td>
<td>108.4 ± 6.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6–11 for both series). Data were obtained from 2 different experimental series: 1) carbamylcholine (CCh) dose-response curves; 2) Ca\(^{2+}\) dose-response curves. Cytosolic Ca\(^{2+}\) level (\([\text{Ca}^{2+}]_c\)) levels (in nM) are provided for 2.5 mM external Ca\(^{2+}\) concentration. Data of \([\text{Ca}^{2+}]_c\), in series 1 were measured before CCh administration. \([\text{Ca}^{2+}]_c\), in series 2 was retrieved from the dose-response curves for 2.5 mM Ca\(^{2+}\); MRA, maximal response amplitude, calculated by fitting the Ca\(^{2+}\) dose-response curves (4); HS, heat stress; C, control; STHA, short-term heat acclimation; LTHA, long-term heat acclimation. *P < 0.05 between pairs LTHA vs. C, LTHA-HS vs. C-HS.

amount of released Ca\(^{2+}\) was normalized for cell number (mg protein in the measured suspension volume). For this purpose, aliquots of the cell suspension were taken before the signal measurement, lysed with 0.1 M NaOH, and protein was determined according to Bradford. \([\text{Ca}^{2+}]_c\) was measured as above.

Glandular output. To characterize the \([\text{Ca}^{2+}]_c\)-submaxillary glandular performance relationship, the correlation between the CCh-evoked \([\text{Ca}^{2+}]_c\) signals obtained in this investigation and glandular performance expressed by specific markers in response to an equimolar CCh stimulation was determined. For this purpose, we resorted to previous databases on dose-response curves for CCh-evoked \(^{86}\)Rb efflux, a marker of CCh-MR coupling (15) and CCh-induced salivation (22). Briefly, \(^{86}\)Rb efflux was measured in a perfusion system using gland slices loaded with \(^{86}\)Rb. For stimulation, slices were superfused with aerated (95% O\(_2\)-5% CO\(_2\)) Krebs buffer containing CCh. \(^{86}\)Rb efflux was measured in the outflow fractions (30). Salivary flow was measured in rats with a chronically cannulated submaxillary gland. For glandular stimulation, the drug was administered as a bolus via the jugular vein. Unfortunately, in the latter experiments, a complete series was available only for nonacclimated and 2 days-acclimated rats (22). In all the experimental series, both glandular physiological performance and \([\text{Ca}^{2+}]_c\) were measured at the same CCh stimulation range.

Calcium quantification, calculations, and statistics. Cytosolic calcium was calculated from the ratio of the fluorescence emitted at 510 nm at the two excitation wavelengths, 340 and 380 nm, using 161 nM as the dissociation constant \((K_d)\) of fura 2 for Ca\(^{2+}\) at 25°C, the temperature at which all measurements were conducted. The \(K_d\) of fura 2 for Ca\(^{2+}\) at this temperature was derived by extrapolation from Grynkiewicz et al. (11) and Haugland (12). The calcium concentration was determined at the end of each individual dose-response curve by measuring the limiting 340/380 ratio for the unbound fura in free calcium medium and the limiting ratio for the bound form at maximal calcium concentration subsequent to cell permeabilization with saponin at the end of each experiment. In the suspension of permeabilized cells, the maximal signal was measured in the presence of 10 mM Ca\(^{2+}\), and the minimal signal was measured by gradual adding of 0.5 M EGTA stock solution to the experimental cuvette. Both the saturating Ca\(^{2+}\) concentration for the limiting bound ratio and the EGTA concentration measured for the limiting ratio of the unbound fura were determined by stabilization of the peak signal and its absolute quenching, respectively.

All dose-response curves were approximated by a generalized Michaelis-Menten model (5), where the external agent at the different experimental series was CCh, IP\(_3\), or external Ca\(^{2+}\). The maximal response to the agonist [maximal response amplitude (MRA)] was obtained by fitting the dose-response curves [modified Lineweaver-Burk plot of the dose-response curve (5)]. The calculated MRA for each individual treatment was used to calculate responses as a percent of the maximal response.

To assess significant changes, ANOVA, Student’s t-test, or nonparametric statistics were used. P < 0.05 was considered significant.

RESULTS

Resting Ca\(^{2+}\) level in the course of heat acclimation. There was no significant change in the resting \([\text{Ca}^{2+}]_c\) in acclimated vs. nonacclimated rats, as reflected in the calcium dose-response curves of the resting glandular cells and in the basal \([\text{Ca}^{2+}]_c\), measured in the CCh dose-response experimental series. However, the basal \([\text{Ca}^{2+}]_c\), measured in the latter experimental series was lower than that measured at a similar (2.5 mM) external calcium concentration in the calcium dose-response experiments (Table 1). The only statistically significant difference among the groups was the coefficient of the equation fitting the dose-response curves, which in the control rats was markedly lower than unity (0.58) and in the LTHA group increased to 0.78.

CCh-evoked Ca\(^{2+}\) signal and IP\(_3\) endoplasmic reticulum Ca\(^{2+}\) stores. As shown in Fig. 1, the response to CCh stimulation was dose dependent. In the nonacclimated group, the maximal response was beyond the
range of the concentrations used. In contrast, during STHA, the maximal CCh-evoked response peaked at 100 mM CCh, and this was 26% lower than in the controls (P<0.02). On LTHA, the [Ca\(^{2+}\)]\(_s\) amplitude returned toward preacclimation level. The MRA was lower than in the nonacclimated group by 19%. No significant changes in EC\(_{50}\) were observed among the groups (Table 2). An example of individual records of the time course of the Ca\(^{2+}\) on CCh addition is provided in Fig. 2. Figure 3 shows the IP\(_3\) receptor-mediated pool size. Heat acclimation decreased the IP\(_3\) receptor-mediated release of calcium from the endoplasmic stores, both at STHA and LTHA. However, the curves presenting the values as percent MRA (Fig. 3, bottom) show right and left shifts in the STHA and LTHA groups, respectively. The EC\(_{50}\) for these groups (Table 2) differed significantly (P<0.05), suggesting differences in the sensitivity of the response to IP\(_3\) between the STHA and the LTHA groups.

Glandular performance: [Ca\(^{2+}\)]\(_s\)-glandular output relationships. Data from our previous studies (16, 22) allow us to correlate glandular performance with the evoked [Ca\(^{2+}\)]\(_s\), an essential step in the mobilization of water secretion. The [Ca\(^{2+}\)]\(_s\)-CCh-stimulated \(^86\)Rb efflux (a marker for agonist-receptor coupling) relationship is demonstrated in Fig. 4. It is evident that during STHA, at the lower [Ca\(^{2+}\)]\(_s\) range (corresponding to a lower CCh dose), the \(^86\)Rb efflux was much smaller than in the control glands. The maximal [Ca\(^{2+}\)]\(_s\) obtained in this group corresponded to supramaximal CCh stimulation and induced an \(^86\)Rb efflux similar to that of the control at a similar [Ca\(^{2+}\)]\(_s\). However, the STHA glands, as opposed to the C glands, were unable to produce greater signals to further increase secretion. LTHA exhibited a biphasic curve: at the lower [Ca\(^{2+}\)]\(_s\) range, a very low \(^86\)Rb efflux was observed. The high-amplitude signals were associated with an \(^86\)Rb efflux markedly exceeding that of the C gland at similar signal amplitudes. This may suggest two distinct receptor populations at this acclimation phase, with a more efficient response in at least one of the receptor populations.

Agonist-induced saliva flow was used as an estimate of glandular secretory processes distal to [Ca\(^{2+}\)]\(_s\) (Fig. 4). The available data are only partial. However, similar to the [Ca\(^{2+}\)]\(_s\)-\(^86\)Rb efflux relationship, during

### Table 2. MRA and EC\(_{50}\) for CCh and IP\(_3\)-evoked [Ca\(^{2+}\)]\(_s\)

<table>
<thead>
<tr>
<th></th>
<th>CCh Evoked, nM</th>
<th>IP(_3) Evoked, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRA EC(_{50})</td>
<td>MRA EC(_{50})</td>
</tr>
<tr>
<td>Control</td>
<td>172.3 ± 16.3</td>
<td>2.95 ± 0.6</td>
</tr>
<tr>
<td>STHA</td>
<td>128.3 ± 11.7*</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>LTHA</td>
<td>139.1 ± 4.3</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>CHS</td>
<td>234.2 ± 6.9*</td>
<td>8.9 ± 0.7*</td>
</tr>
<tr>
<td>STHA-HS</td>
<td>125.4 ± 14.0</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>LTHA-HS</td>
<td>130.5 ± 18.1</td>
<td>3.7 ± 1.7</td>
</tr>
</tbody>
</table>

Values are average ± SE. MRA values were calculated by fitting the dose-response curves. \*Significant difference from the controls; \*\*significant difference from STHA; significant difference from the matched nonheat-stressed group; \*\*\*P < 0.05, \*P < 0.01. For details, see Figs. 1, 3, 6, 7, top.
STHA, greater $[Ca^{2+}]_s$ increments were required to produce similar saliva flows. Unfortunately, such data are not available for the LTHA phase.

Heat stress superimposed on heat acclimation. Acute heat stress reduced resting $[Ca^{2+}]_c$ in the LTHA glands when external $Ca^{2+}$ was gradually raised (Fig. 5). The MRA of the $[Ca^{2+}]_c$ in the LTHA cells subjected to increasing calcium concentrations was $109.1 \pm 2.4$ nM in the LTHA group vs. $150.1 \pm 11.6$ nM in the C group. The right shift of the curve showing the values as percent MRA (Fig. 5, bottom) indicates decreased sensitivity of the LTHA cells to external calcium loads. During heat stress, in nonacclimated glands CCh-evoked $[Ca^{2+}]_s$ was markedly higher than in the nonheat-stress-matched group (Fig. 6), and EC$_{50}$ was significantly higher ($8.9 \pm 0.7$ vs. $2.95 \pm 0.63$, $0.01 < P < 0.05$, for nonacclimated heat-stressed vs. nonacclimated glands, respectively). In contrast, the acclimated cells did not differ significantly from the nonstressed cells (not shown). IP$_3$-sensitive stores dropped significantly in the nonacclimated groups. In the LTHA glandular cells, the IP$_3$-sensitive store was markedly elevated with a concomitant decrease in the sensitivity of the response to IP$_3$ stimulation compared with the nonheat stress condition in this group (Fig. 7). The difference in EC$_{50}$ for the two groups was significant (LTHA-HS: $6.66 \pm 2.37$; LTHA 3.23 $\pm 1.24$; $P < 0.05$).

**DISCUSSION**

Our present results support our previous studies indicating that the muscarinic signaling for water secretion undergoes temporal changes during heat acclimation and the amplitude of the evoked $[Ca^{2+}]_s$ is the target for both chronic and acute thermal effects. During heat acclimation, $[Ca^{2+}]_s$ undergoes biphasic changes, and the mechanisms leading to these changes differ in the two studied phases. During the STHA phase, $[Ca^{2+}]_s$ is reduced coincidentally with a drop in glandular output. On LTHA, the high-amplitude $[Ca^{2+}]_s$ (Fig. 4) induces a greater glandular response than in nonacclimated glands, suggesting that processes other than calcium mobilization contribute to this beneficial effect. These responses are heat acclimation specific and override opposing heat stress-induced effects such as augmented CCh-induced $[Ca^{2+}]_s$,...
which, in terms of the agonist-mediated signal, are only seen in the nonacclimated rats.

Resting calcium level. The basal calcium level in the resting exocrine gland is subject to changes via alterations in both the cell membrane and the intracellular stores (e.g., 10, 21, 25, 31, 34). Although there were no significant differences between the various experimental groups, the contribution of the controlling mechanism varied somewhat because they appeared to have different kinetics, as inferred from Table 1. These mechanisms are beyond the scope of our investigation (it is noteworthy that the resting [Ca$^{2+}$]c of cells exposed to progressively elevated calcium concentrations was higher than that of cells maintained at 2.5 mM calcium throughout the experiments). We hypothesize that this phenomenon stems from an altered load on the plasma cell-membrane pumps in the two experimental conditions.

Calcium signals. In the present investigation, the size of the IP$_3$-sensitive calcium pool size and the magnitude of the membrane receptor-mediated calcium signal were measured. The qualitative effects of heat acclimation on these variables are presented in Table 3. During the transient STHA phase, CCh-evoked [Ca$^{2+}$]$_i$ was markedly reduced with no change in the sensitivity of the response. Concomitantly, IP$_3$-sensitive endoplasmic stores were significantly reduced, together with a decrease in the sensitivity of the response to IP$_3$. Previous studies reported decreased affinity of the MR to the agonist on STHA (15), due, at least in part, to the effects of acclimation on the receptor-coupled G protein (19). This finding may localize the basolateral cell membrane as the first site at which, during STHA, impairment in the muscarinic transduction pathway takes place, leading to dimin-

<table>
<thead>
<tr>
<th>STHA</th>
<th>LTHA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Affinity</td>
<td>↓</td>
<td>NC</td>
</tr>
<tr>
<td>CCh-evoked [Ca$^{2+}$]$_i$</td>
<td>↓</td>
<td>NC</td>
</tr>
<tr>
<td>IP$_3$-sensitive Ca$^{2+}$ stores</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Sensitivity of IP$_3$-induced response</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Glandular performance</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kloog et al. (22)</td>
</tr>
</tbody>
</table>

The changes presented are relative to the nonacclimated group. ↑, Downregulation; ↑, upregulation; NC, no change.
ished IP$_3$ production and decreased release of calcium from the endoplasmic stores. Several steps in this cascade were reported by Fujinami et al. (9) in the parotid gland.

On LTHA, CCh-evoked [Ca$_{2+}$]$_s$ tended to revert to the preacclimation level. At this acclimation stage, upregulation of MR with preacclimation affinity had taken place (15). Because there was no rise in [Ca$_{2+}$]$_s$, it was suggested that the drop in IP$_3$-sensitive calcium stores (compared with the nonacclimated condition) blunts the increase in [Ca$_{2+}$]$_s$ amplitude to levels exceeding that of the nonacclimated signal. This conclusion is supported by the finding that the CCh-evoked [Ca$_{2+}$]$_s$ at zero extracellular calcium level (Fig. 1, inset) diminished by the same order of magnitude in the C and the LTHA groups, suggesting that the contribution of extracellular calcium to the evoked [Ca$_{2+}$]$_s$ (4, 18, 24, 27, 29, 34) is similar for both groups. It is noteworthy that the left shift of the IP$_3$ dose-response curve for the LTHA group may imply increased sensitivity of the response to IP$_3$, allowing the observed faster response on agonist stimulation.

In conclusion, it is likely that during STHA, decreased MR affinity and reduced IP$_3$-sensitive calcium pool size contribute to the smaller STHA [Ca$_{2+}$]$_s$, whereas recovery of the [Ca$_{2+}$]$_s$ on LTHA depends mainly on alterations in the plasma membrane. Temporal changes in unsaturated fatty acids and free cholesterol levels on heat acclimation, leading to altered plasma membrane properties and function, were recently measured in salivary glands of short- and long-term heat-acclimated rats (H. Shmida, P. Kaspler, M. Horowitz, and Y. Barenholz, unpublished observations). These changes may account for the resumed preacclimation affinity of the MR in the LTHA glands.

The evocation of [Ca$_{2+}$]$_s$ activates K$^+$ and Cl$^-$ channels in the basolateral and luminal cell membranes, respectively. The result is a net loss of these ions, water mobilization into the acinar lumen, and cell shrinkage. The correlation between [Ca$_{2+}$]$_s$ and the efflux of K$^+$, Cl$^-$, or salivary flow may shed light on the polarity of the acclimatory responses in this water secretion pathway and indicate whether processes other than [Ca$_{2+}$]$_s$ amplitude are responsible for glandular function in the course of heat acclimation. The relationship between [Ca$_{2+}$]$_s$ and K$^+$ marker efflux, which signifies receptor activation, as well as the [Ca$_{2+}$]$_s$-saliva flow relationship (Fig. 4), implies that at equimolar CCh stimulation there is no correlation between the amplitude of the [Ca$_{2+}$]$_s$ produced and glandular performance among the nonacclimating and acclimating groups. At the range of the higher [Ca$_{2+}$]$_s$ produced in LTHA glands, the glandular output-to-[Ca$_{2+}$]$_s$ ratio was greater in the LTHA glands than in the nonacclimated glands for similar [Ca$_{2+}$]$_s$, suggesting increased efficiency of processes distal to the [Ca$_{2+}$]$_s$, on acclimation.

STHA glands fail to produce high-amplitude [Ca$_{2+}$]$_s$. In this group, the slope of the [Ca$_{2+}$]$_s$-saliva flow relationship is similar to that of the control, implying that the decreased output is associated, at least partially, with lowered [Ca$_{2+}$]$_s$. This may be associated with decreased CCh-induced IP$_3$ production or decreased IP$_3$-induced response. Our previous studies (e.g., Ref. 15) indicated lower MR affinity. However, because there was no change in EC$_{50}$ in the CCh dose-response curves, it is likely that during STHA IP$_3$ production is the rate-limiting step in glandular performance.

Effect of heat stress. During heat stress, the acquired heat acclimation advantage of the LTHA cells is the avoidance of elevated cytosolic calcium. This was manifested in the present investigation by the reduced basal [Ca$_{2+}$]$_s$ and by its attenuated rise with an increasing calcium load. This fits many other observations from our laboratory on altered features of the cell membrane in heat-acclimated glands as well as in cardiomyocytes (H. Shmida et al., unpublished observations) possibly blunting stress-induced calcium overload (M. Horowitz and R. Shlomai, unpublished observations).

On glandular stimulation, heat stress accelerates Ca$_{2+}$ influx and mobilizes intracellular calcium stores, leading to a rise in intracellular Ca$_{2+}$ level due to unsynchronized inhibition of adenylate cyclase and phosphatidylinositol-2-phosphate signaling pathways (1, 2, 3, 20). Our data on the heat-stressed control group are in accord with these findings, as manifested by the marked elevation in the amplitude of the [Ca$_{2+}$]$_s$. This elevation can be partially explained by the marked increase in MR affinity (15), possibly leading to augmented IP$_3$ production, as shown for heat-stressed cells by several investigators (35) and by increased activity of the Na/Ca exchanger, as shown for human endothelial cells (23). It does not tally, however, with the observed decrease in the IP$_3$-sensitive calcium pool, implying the contribution of extracellular calcium to the signal produced. Collectively, glandular performance, as indicated by $^{86}$Rb efflux (15), was impaired.

In contrast to the nonacclimated heat-stressed rats, the heat-acclimated heat-stressed groups did not differ in their CCh-evoked [Ca$_{2+}$]$_s$ from the nonstressed, with lowered [Ca$_{2+}$]$_s$. This may be associated with decreased CCh-induced IP$_3$ production or decreased IP$_3$-induced response. Our previous studies (e.g., Ref. 15) indicated lower MR affinity. However, because there was no change in EC$_{50}$ in the CCh dose-response curves, it is likely that during STHA IP$_3$ production is the rate-limiting step in glandular performance.

Effect of heat stress. During heat stress, the acquired heat acclimation advantage of the LTHA cells is the avoidance of elevated cytosolic calcium. This was manifested in the present investigation by the reduced basal [Ca$_{2+}$]$_s$ and by its attenuated rise with an increasing calcium load. This fits many other observations from our laboratory on altered features of the cell membrane in heat-acclimated glands as well as in cardiomyocytes (H. Shmida et al., unpublished observations) possibly blunting stress-induced calcium overload (M. Horowitz and R. Shlomai, unpublished observations).

On glandular stimulation, heat stress accelerates Ca$_{2+}$ influx and mobilizes intracellular calcium stores, leading to a rise in intracellular Ca$_{2+}$ level due to unsynchronized inhibition of adenylate cyclase and phosphatidylinositol-2-phosphate signaling pathways (1, 2, 3, 20). Our data on the heat-stressed control group are in accord with these findings, as manifested by the marked elevation in the amplitude of the [Ca$_{2+}$]$_s$. This elevation can be partially explained by the marked increase in MR affinity (15), possibly leading to augmented IP$_3$ production, as shown for heat-stressed cells by several investigators (35) and by increased activity of the Na/Ca exchanger, as shown for human endothelial cells (23). It does not tally, however, with the observed decrease in the IP$_3$-sensitive calcium pool, implying the contribution of extracellular calcium to the signal produced. Collectively, glandular performance, as indicated by $^{86}$Rb efflux (15), was impaired.

In contrast to the nonacclimated heat-stressed rats, the heat-acclimated heat-stressed groups did not differ in their CCh-evoked [Ca$_{2+}$]$_s$ from the nonstressed, with lowered [Ca$_{2+}$]$_s$. This may be associated with decreased CCh-induced IP$_3$ production or decreased IP$_3$-induced response. Our previous studies (e.g., Ref. 15) indicated lower MR affinity. However, because there was no change in EC$_{50}$ in the CCh dose-response curves, it is likely that during STHA IP$_3$ production is the rate-limiting step in glandular performance.

Effect of heat stress. During heat stress, the acquired heat acclimation advantage of the LTHA cells is the avoidance of elevated cytosolic calcium. This was manifested in the present investigation by the reduced basal [Ca$_{2+}$]$_s$ and by its attenuated rise with an increasing calcium load. This fits many other observations from our laboratory on altered features of the cell membrane in heat-acclimated glands as well as in cardiomyocytes (H. Shmida et al., unpublished observations) possibly blunting stress-induced calcium overload (M. Horowitz and R. Shlomai, unpublished observations).

On glandular stimulation, heat stress accelerates Ca$_{2+}$ influx and mobilizes intracellular calcium stores, leading to a rise in intracellular Ca$_{2+}$ level due to unsynchronized inhibition of adenylate cyclase and phosphatidylinositol-2-phosphate signaling pathways (1, 2, 3, 20). Our data on the heat-stressed control group are in accord with these findings, as manifested by the marked elevation in the amplitude of the [Ca$_{2+}$]$_s$. This elevation can be partially explained by the marked increase in MR affinity (15), possibly leading to augmented IP$_3$ production, as shown for heat-stressed cells by several investigators (35) and by increased activity of the Na/Ca exchanger, as shown for human endothelial cells (23). It does not tally, however, with the observed decrease in the IP$_3$-sensitive calcium pool, implying the contribution of extracellular calcium to the signal produced. Collectively, glandular performance, as indicated by $^{86}$Rb efflux (15), was impaired.

In contrast to the nonacclimated heat-stressed rats, the heat-acclimated heat-stressed groups did not differ in their CCh-evoked [Ca$_{2+}$]$_s$ from the nonstressed,
heat-acclimated rats. Different mechanisms, however, contributed to this apparent stability in the two acclimation phases. In the STHA glands, neither of the factors studied differed from those of the nonstressed matching group, suggesting that changes occurring during the STHA state override the effects of heat stress. In contrast, on LTHA, despite implications of augmented IP$_3$-sensitive Ca$^{2+}$ stores, the sensitivity of the IP$_3$ response decreased. Qualitative presentation of changes in MR signaling before and after heat stress with heat acclimation is presented in Table 4.

Taken together, our present data, as well as that from a previous study (15) and unpublished observations (P. Kaspler and M. Horowitz), suggest that heat acclimation and heat stress produce a diametrically opposing effect on the MR signaling cascade. In nonacclimated rats, the effects of heat stress are primarily intracellular and downstream of the MR. On acclimation, however, adaptive changes take place both in the plasma membrane MR and intracellularly. These counter protection against the deleterious effects of heat stress on calcium homeostasis. Interestingly, such changes, particularly with respect to calcium turnover, have been observed in thermotolerant cell lines as well (20).

**Perspectives**

Calcium homeostasis is a major factor in cellular integrity. Our findings that calcium signaling provides a target for long-standing acclamatory responses en-gender new concepts and merit a large-scale study of calcium compartmentalization (e.g., caffeine-sensitive stores) and calcium trafficking and its regulation. The possibility of molecular changes leading to altered expression of the targeted proteins involved in calcium signaling is still an open question as well. Studies from our laboratory on calcium regulation in the heart support this notion.

**REFERENCES**


