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Acetylcholine-induced metabolic changes in the perfused rabbit mandibular salivary gland studied by ^{31}P-NMR spectroscopy

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Changes in the content of high-energy phosphates, intracellular pH (pH_i) and the ratio of MgATP to total ATP ([MgATP]/[ATP]$_t$) resulting from continuous stimulation with acetylcholine (10^{-9} to 10^{-4} M) were measured by ^{31}P-NMR spectroscopy in the isolated, perfused rabbit mandibular gland at 37°C. With 10^{-9} to 10^{-7} M acetylcholine, no significant changes in these parameters were observed. On stimulation with 10^{-6} M acetylcholine, the optimal concentration for sustained secretion, the content of ATP decreased by $28 \pm 10\%$ (mean ± S.E.; $n = 8$) of its control value. pH_i decreased initially by approx. 0.05 pH unit, then showed an alkalinization of 0.09 ± 0.02 pH unit ($n = 8$). With 10^{-5} and 10^{-4} M acetylcholine, changes in ATP and pH_i were similar to those induced by 10^{-6} M acetylcholine: the total content of high-energy phosphates remained at approx. 70% of the control value and no decrease in [MgATP]/[ATP]$_t$ was observed. As possible causes of the reduced secretory rate observed with higher concentrations of acetylcholine (10^{-5} to 10^{-3} M), we can exclude depletion of high-energy phosphates, inhibition of metabolism caused by intracellular acidosis, and inhibition of ATP usage caused by a decrease in MgATP availability.

Introduction

Continuous stimulation of the isolated, perfused rabbit mandibular salivary gland with the muscarinic agonist acetylcholine elicits a characteristic pattern of fluid secretion. With the optimal concentration of acetylcholine for fluid secretion (10^{-6} M), an initially brisk secretion declines over a period of about 15 min to a plateau value which may be sustained for 2 h or longer [1]. With higher concentrations of acetylcholine (10^{-5} to 10^{-3} M), although the initial secretory rate is similar or even slightly greater, the decline in flow (tachyphylaxis) is more pronounced and the plateau secretory rate is correspondingly reduced [2].

There are many possible explanations for the phenomenon of tachyphylaxis and the reduced response to higher concentrations of acetylcholine. Case et al. [1] listed as possibilities: (1) receptor desensitization, (2) changing intracellular ion concentrations, (3) intracellular feedback inhibition by second messengers, and (4) exhaustion of metabolic stores. Since the effect is particularly pronounced in the isolated perfused gland pre-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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paration, metabolic exhaustion due to inadequate perfusion cannot be excluded as a contributory factor. However, Murakami et al. [3] recently reported that isotonic replacement of Na^+ by 46 mM choline evokes near-maximal secretion from the perfused mandibular gland of the rat without inducing tachyphylaxis. They were, therefore, able to exclude metabolic exhaustion as a possible cause in the rat.

In this study, we have used ^{31}P nuclear magnetic resonance (NMR) spectroscopy to examine possible metabolic causes of tachyphylaxis in the rabbit mandibular gland including: (1) exhaustion of high-energy phosphates, (2) inhibition of metabolism due to intracellular acidosis resulting from anaerobic metabolism, and (3) inhibition of ATP usage due to decreased availability of MgATP. Changes in the content of high-energy phosphorus compounds, the intracellular pH (pH_i) and the ratio of MgATP to total ATP have been measured in the isolated, perfused rabbit mandibular gland during continuous stimulation with acetylcholine at concentrations ranging from 10^{-9} to 10^{-4} M.

Materials and Methods

Female half-lop rabbits weighing 2.0–2.5 kg were anaesthetized by intraperitoneal injection of urethane (1.5 g/kg body weight). Mandibular glands (wet weight approx. 0.4 g) were isolated and cannulated for vascular perfusion as described previously [1]. They were perfused at a constant rate of 4 ml/min using a peristaltic pump (Watson Marlow). The composition of the perfusate (in mM) was: Na^+, 145.0; K^+, 4.5; Cl^-, 125.0; Ca^{2+}, 1.5; Mg^{2+}, 1.0; SO_4^{2-}, 1.0, HCO_3^- 25.0, Hepes, 5.0; and glucose, 5.0. The solution was pre-heated to 37 °C and equilibrated with 5% CO_2 in O_2 to give a final pH of 7.4. The perfused gland was mounted in a glass sample tube (internal diameter 18 mm) and simultaneously superfused at 8 ml/min with preheated saline solution (0.9% NaCl) to remove the effluent perfusate and secreted saliva.

The perfusion line from the pump to the gland was approx. 1.5 m in length. In order to avoid any loss of heat and/or dissolved gases from the perfusate, the perfusion line consisted of a fine polythene tube (internal diameter 1.0 mm) concentrically enclosed within PVC tubing carrying a steady flow of O_2/CO_2 mixture. This in turn was enclosed within larger bore PVC tubing carrying water pre-heated to 37 °C. Inside the bore of the magnet, the perfusate and the saline superfusate were subjected to a final stage of temperature adjustment as they passed through a two-channel water-jacketed glass coil immediately adjacent to the tissue chamber.

The content of phosphorus-containing metabolites in the gland, the intracellular pH and the ratio of MgATP to total ATP ($[MgATP]/[ATP]_t$) were determined by ^{31}P-NMR spectroscopy [4,5]. ^{31}P-NMR spectra were collected at 81.1 MHz on a BIOSPEC 150/4.7 spectrometer (Oxford Research Systems, Abingdon) using a home-built ^{31}P probe with a 25 mm diameter, four-turn solenoidal transmitter/receiver coil of 3 mm copper wire.

Radio frequency pulses of 10–12 μs (45° pulse angle) were used with a pulse repetition time of 0.693 s, a spectral width of 5 kHz, and a spectral data size of 2048 points. Fourier transformation was performed with exponential line broadening, usually of 10–15 Hz, after extending the spectral data size to 4096 points. Resonances were assigned from their chemical shifts by comparison with those of pure samples. Chemical shifts were expressed relative to the chemical shift of intracellular creatine phosphate (0.0 ppm).

The gland content of each phosphorus metabolite was determined from the area of its resonance, using the appropriate correction for partial saturation (see Results). The content of ADP was calculated by subtracting the area of the βATP resonance from that of the γATP/βADP resonance. The ADP content was not calculated from the creatine kinase equilibrium because studies in the rat submandibular gland have indicated that in that tissue there are three different types of creatine kinase, each with different kinetic parameters, and that much of the enzyme activity is localized in the mitochondria [6].

pH_i was calculated from the chemical shift of inorganic phosphate (δ_0) [7] and is expressed as the difference (ΔpH_i) from the resting value using the following formula:

$$\Delta pH_i = \log \frac{\delta_0 - \delta_1}{\delta_2 - \delta_0} - \log \frac{\delta_r - \delta_1}{\delta_2 - \delta_r}$$

where δ_r is the chemical shift of inorganic phosphate in the unstimulated gland. For the chemical shifts of $H_2PO_4^-$ (δ_1) and HPO_4^{2-} (δ_2), measured values of 3.15 and 5.73 ppm were used, respectively.

$[MgATP]/[ATP]_t$ was calculated from the chemical shift of the β-phosphate resonance of ATP (δ_β) [8,9] using:

$$\frac{[MgATP]}{[ATP]_t} = \frac{\delta_\beta - \delta_{ATP}}{\delta_{MgATP} - \delta_{ATP}}$$

The measured chemical shifts of the β-phosphate groups of ATP (δ_{ATP}) and MgATP (δ_{MgATP}) used for this calculation were -18.93 and -15.85 ppm, respectively.

After a period of control perfusion lasting 60 min, changes in the ^{31}P spectrum of the gland were assessed during a 30-min stimulation period with acetylcholine (10^{-9} to 10^{-4} M) and then for a further 30 or 40 min. Spectra were obtained over 2.5 min periods (208 scans) during the initial 10 min of stimulation with acetylcholine, and over 5 min periods (416 scans) thereafter. Each gland was used for one or two experiments and the same protocol was followed in each experiment. Four to eight experiments were performed with each concentration of acetylcholine.

All values are expressed as the mean and standard error of the mean (mean ± S.E.). Statistical analyses were performed using Student's *t*-test, and *P* values less than 0.05 were accepted as significant.

Results

Fluid secretion during supramaximal acetylcholine stimulation

Fig. 1 shows the secretory response of the perfused rabbit mandibular gland to continuous stimulation with acetylcholine at optimal ($0.8 \cdot 10^{-6}$ M) and supramaximal ($0.8 \cdot 10^{-5}$ and $0.8 \cdot 10^{-4}$ M) concentrations (unpublished data from a previous study [2]). The mean flow rate during the period 10–30 min following the onset of stimulation was 59.3 ± 5.9 µl/min ($n = 8$) with $0.8 \cdot 10^{-6}$ M acetylcholine. With the higher concentrations of acetylcholine, the fluid output over this period was significantly reduced: 39.3 ± 3.3 µl/min ($n = 8$, $P < 0.02$) at $0.8 \cdot 10^{-5}$ M and 31.1 ± 2.8 µl/min ($n = 8$, $P < 0.002$) at $0.8 \cdot 10^{-4}$ M.

^{31}P-NMR spectrum of the perfused rabbit mandibular gland

The ^{31}P-NMR spectrum of the perfused rabbit mandibular gland consists of eight principal reso-

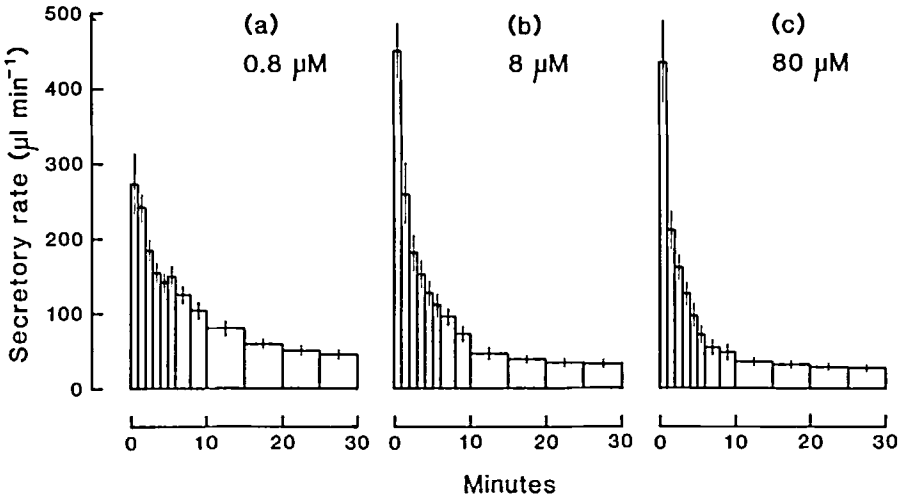

Fig. 1. Secretory flow as a function of time during continuous stimulation of the isolated, perfused rabbit mandibular gland with optimal ((a) $0.8 \cdot 10^{-6}$ M) and two supramaximal concentrations of acetylcholine ((b) $0.8 \cdot 10^{-5}$ and (c) $0.8 \cdot 10^{-4}$ M). The flow curves were compiled using unpublished data from a previous study [2], and are presented as means ± S.E. from eight glands. Perfusion conditions were the same as those used in the NMR studies except that Hepes was absent from the perfusate.

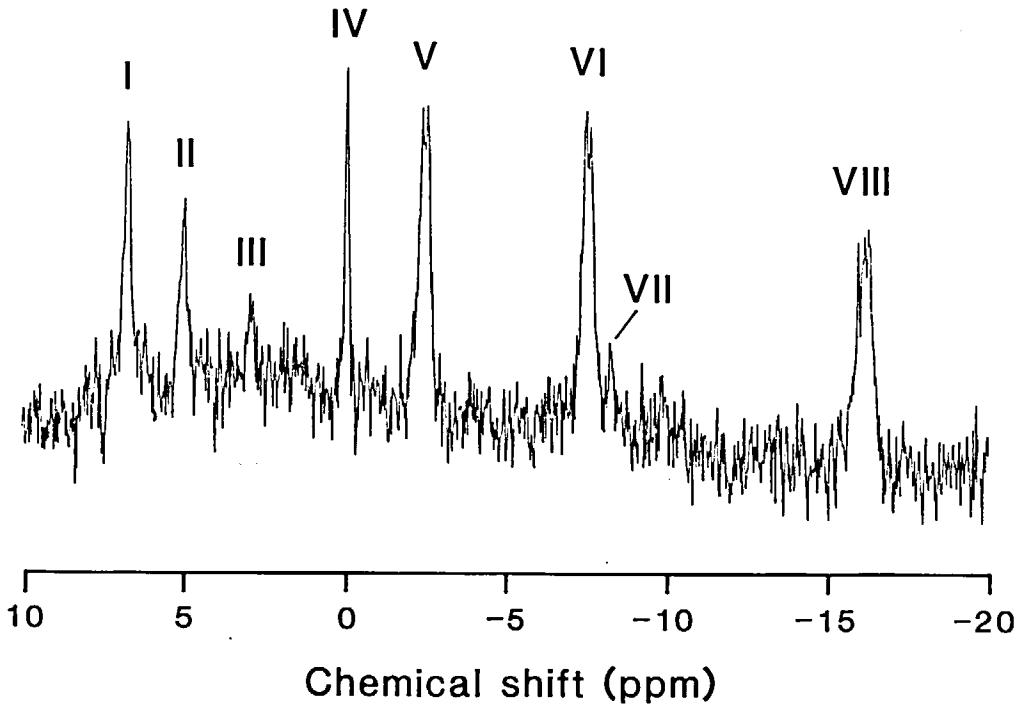

Fig. 2. ^{31}P-NMR spectrum of the perfused mandibular gland of the rabbit at 37°C. Eight resonances were identified: I, phosphomonoesters (6.8 ppm) and AMP (6.5 ppm); II, inorganic phosphate (P_i, 5.0 ppm); III, phosphodiesters (3.0 ppm); IV, creatine phosphate (PCr, 0.0 ppm); V, γ-phosphate of ATP and β-phosphate of ADP (γATP/βADP, −2.4 ppm); VI, α-phosphate of ATP and α-phosphate of ADP (αATP/αADP, −7.5 ppm); VII, NAD^+ and NADH (NAD, −8.5 ppm): and VIII, β-phosphate of ATP (βATP, −16.0 ppm). In order to obtain this high resolution spectrum, in which spin-spin coupling of the ATP peaks is evident, only 1 Hz of line broadening was applied.

nances (Fig. 2). These have been assigned as follows: I, phosphomonoesters including AMP; II, inorganic phosphate; III, phosphodiesters; IV, creatine phosphate; V, γ-phosphate of ATP and β-phosphate of ADP; VI, α-phosphate of ATP and α-phosphate of ADP; VII, NAD^+ and NADH; and VIII, β-phosphate of ATP. These assignments are consistent with those reported for the dog and rat mandibular glands [4,5], where chemical analysis has confirmed that the bulk of the nucleotide pool is due to adenine nucleoside phosphates with a small component due to guanosine (16% in the rat [5]).

Because of the relatively low sensitivity of the ^{31}P nucleus, the principal acquisition parameters – the pulse angle (θ) and the pulse repetition time (τ) – were set so as to maximize the signal-to-noise ratio of the spectrum. Under these conditions, partial saturation of the resonances occurs, and the relative areas of the resonances do not correspond directly to the relative concentrations of the compounds in the tissue. Each resonance is suppressed by the factor:

$$\frac{[1-\exp(-\tau/T_1)]\sin\theta}{1-\cos\theta\exp(-\tau/T_1)}$$

where T_1 is the spin-lattice relaxation time of that resonance [10]. In order to correct for this effect, T_1 values were determined for each of the resonances in four perfused mandibular glands using the DESPOT (driven equilibrium single-pulse observation of T_1) pulse sequence [10,11]. 1024 scans were accumulated for each of six pulse angles, ranging from 15° to 90° in 15° steps, with a pulse repetition time of 1.0 s. The results are shown in Table I.

The relative concentrations of the different phosphorus metabolites in the unstimulated gland, also listed in Table I, were calculated from the areas of the resonances after correction for satura-

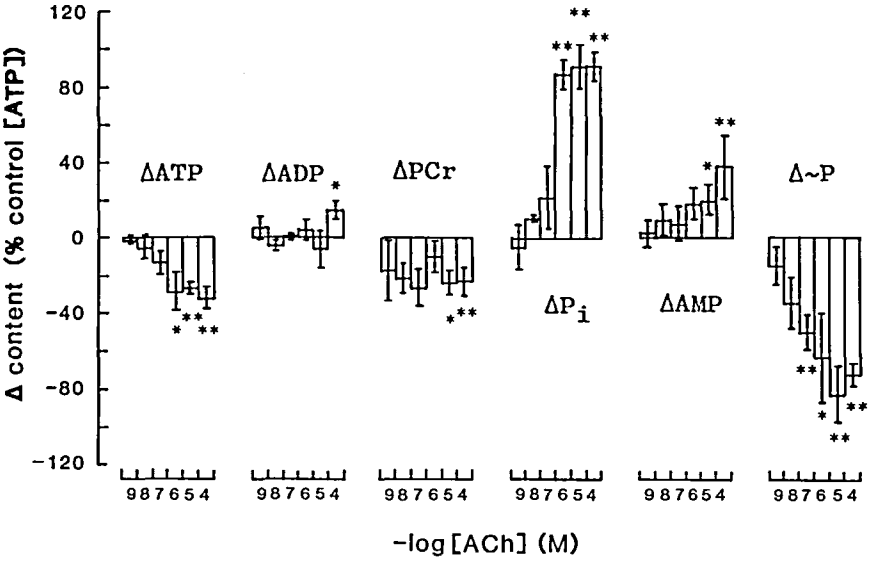

Fig. 3. Concentration dependence of the changes caused by acetylcholine (ACh) in the content of ATP (ΔATP), ADP (ΔADP), PCr (ΔPCr), P_i (ΔP_i), AMP (ΔAMP) and total high-energy phosphates (Δ ~ P) in the perfused rabbit mandibular gland at 37 °C. ^{31}P spectra were summed in individual experiments over the period 10–30 min after the onset of acetylcholine stimulation, and the changes in content were calculated by subtraction of the pre-stimulation control values and expressed as a percentage of the control value for [ATP]. ΔAMP was calculated on the assumption that the change in the phosphomonoester peak was due entirely to a change in AMP content (see Discussion). Δ ~ P was calculated as ΔPCr + ΔADP + 2ΔATP. The data are presented as the means ± S.E. obtained from four to eight different glands. Statistically significant changes are indicated by asterisks: * $P < 0.05$ and ** $P < 0.01$.

tion using the measured T_1 values. The results are expressed as percentages of the ATP content. Under the conditions employed in these studies, the signal intensity of the ATP resonances remained constant for at least 4 h.

Effects of acetylcholine stimulation on phosphorus metabolites

Changes in the content of ATP, ADP, PCr, P_i and phosphomonoester/AMP caused by continuous stimulation with acetylcholine are shown in Fig. 3 as a function of acetylcholine concentration. As shown in Fig. 1, the secretory rate in this preparation declines rapidly over the first 10 min of stimulation to a more sustained, plateau level of secretion. Fig. 3 shows the changes in the content of the phosphorus metabolites, averaged over the period 10–30 min after the onset of stimulation, compared with the pre-stimulation control period. The data thus reflect the state of the gland during the first part of the plateau phase of secretion. The time course of the changes in ATP and phosphomonoester/AMP content are shown in Figs. 4 and 5, respectively.

Stimulation with 10^{-9} to 10^{-8} M acetylcholine evokes minimal secretion of saliva in this preparation [1]. During the application of acetylcholine at these concentrations, and following its withdrawal, the content of ATP, PCr, P_i and phosphomonoester/AMP showed no significant change compared with their control values. The concentration of acetylcholine which evokes half-maximal secretion (10^{-7} M) also caused minimal changes in ATP, PCr and P_i, suggesting that the utilization of ATP could be compensated for almost completely by ATP production in the gland.

With the optimal concentration of acetylcholine for sustained fluid secretion (10^{-6} M), ATP decreased and P_i increased substantially during the first 10 min of stimulation and then remained at a steady value until acetylcholine was withdrawn from the perfusate. During this steady-state period, ATP was reduced by 28 ± 10% and P_i was increased by 87 ± 7% with reference to the ATP

TABLE I

T_1 VALUES AND RELATIVE CONCENTRATIONS OF PHOSPHORUS COMPOUNDS IN THE UNSTIMULATED RABBIT MANDIBULAR GLAND

Spin-lattice relaxation times (T_1) were determined in four unstimulated, isolated, perfused rabbit mandibular glands using the DESPOT pulse sequence. Relative concentrations with reference to the βATP resonance were calculated from the areas of the resonances in ^{31}P-NMR spectra from 17 glands following correction for saturation. Values are presented as the means ± S.E.

	T_1 (s)	Relative concentration (%)
Phosphomonoesters	3.68 ± 0.50	174 ± 12 [a]
Inorganic phosphate	1.44 ± 0.27	32 ± 3
Phosphodiesters	7.58 ± 2.28	–
Creatine phosphate	5.37 ± 0.40	64 ± 5
γATP/βADP	0.96 ± 0.15	–
βATP	1.07 ± 0.09	100
ADP		2 ± 4 [b]
Total high-energy phosphates		266 ± 8 [c]

[a] Calculated on the assumption that the AMP content of the unstimulated gland was negligible.

[b] Calculated by subtracting the area of the βATP resonance from that of the γATP/βADP resonance (see Materials and Methods).

[c] Calculated as 2·[ATP] + [ADP] + [PCr].

content in the unstimulated gland ($n = 8$). Compared with the dog and rat mandibular glands [4,5], the changes in PCr and ADP were noticeably smaller in the rabbit gland.

On stimulation with supramaximal doses of acetylcholine (10^{-5} and 10^{-4} M), which give a reduced secretory rate during the plateau phase (Fig. 1), the initial changes in ATP, PCr and P_i were slightly faster than those caused by 10^{-6} M acetylcholine. During the steady-state period, ATP and PCr were reduced by approx. 35% and 30% with reference to the control value for ATP. The reduction in the total content of high-energy phosphate (~ P) was not significantly different from that caused by 10^{-6} M acetylcholine.

Effects of acetylcholine stimulation on intracellular pH and MgATP

The differences in the values of pH_i and $[MgATP]/[ATP]_t$ during the plateau phase of secretion (10–30 min after the onset of stimulation) compared with their resting values are illustrated in Fig. 6. The time course of the change in pH_i with three different acetylcholine concentrations (10^{-7}, 10^{-6} and 10^{-4} M) is shown in Fig. 7.

The chemical shift of P_i in the unstimulated gland was 5.04 ± 0.01 ppm ($n = 17$). If the dissociation constant of P_i in the cells is assumed to be 6.8 [7] (there is an uncertainty of approx. ±0.1 in this assumption), then the shift corresponds to an intracellular pH of 7.25 ± 0.01 ($n = 17$). Application of acetylcholine at concentrations in the range 10^{-9} to 10^{-7} M caused no significant change in pH_i compared with the unstimulated control value. During stimulation with 10^{-6} M acetylcholine, the optimal dose for sustained fluid secretion, pH_i

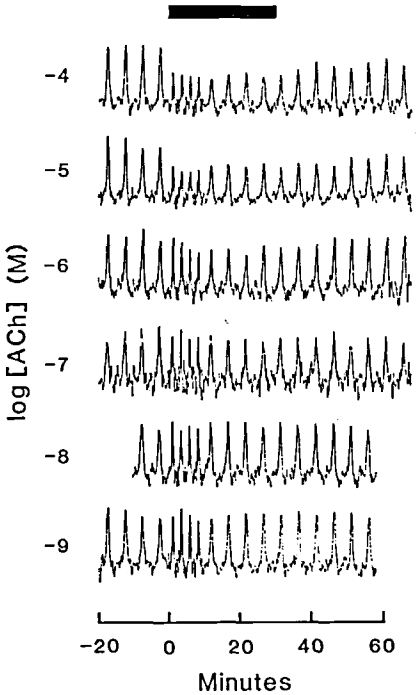

Fig. 4. Time course of the changes in ATP content of the perfused rabbit mandibular gland during stimulation with a range of concentrations of acetylcholine (ACh). βATP resonances from ^{31}P spectra obtained over 5- or 2.5-min periods are plotted sequentially as a function of time. Although the content of ATP is most closely related to the area of the resonance, changes in the height of the resonance also provide a good indication of the changes in ATP content. Experiments were performed using the same protocol in four to six different glands and spectra from corresponding time periods were summed for clarity. The bar at the top of the figure indicates the period of acetylcholine administration.

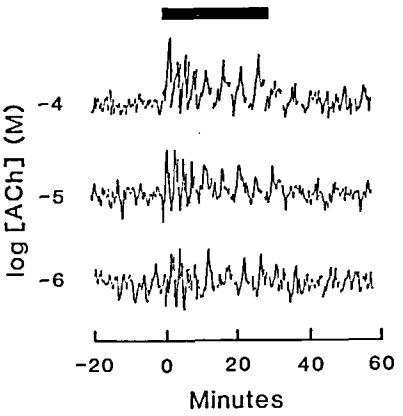

Fig. 5. Time course of the changes in the content of phosphomonoesters and AMP in the perfused rabbit mandibular gland during stimulation with a range of concentrations of acetylcholine (ACh). Changes in the intensity of the phosphomonoester/AMP resonance are shown as the difference spectrum after subtraction of the pre-stimulation control spectrum. An upward deflection represents an increase in phosphomonoester/AMP. The chemical shift of the increased resonance coincided with that of AMP. Experiments were performed using the same protocol in four to six different glands and spectra from corresponding time periods were summed for clarity. The bar at the top of the figure indicates the period of acetylcholine administration.

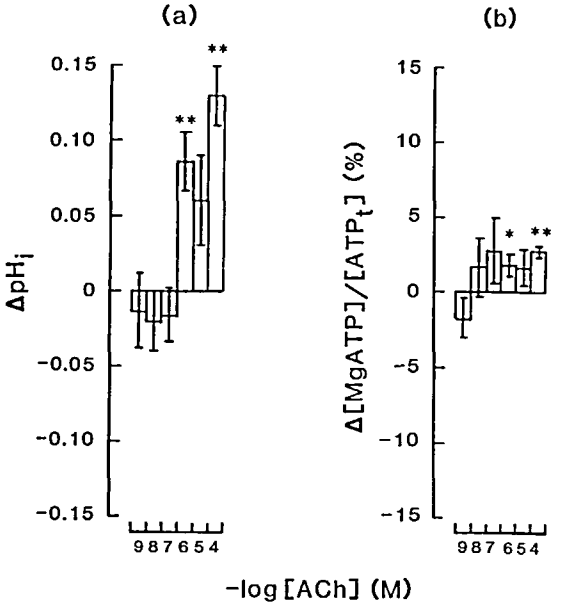

Fig. 6. Changes in (a) intracellular pH and (b) the ratio of MgATP to total ATP in the perfused rabbit mandibular gland following stimulation with a range of concentrations of acetylcholine (ACh). The data are average values for the period 10–30 min after the onset of stimulation compared with the pre-stimulation control period, and are presented as the means ±S.E. obtained from four to eight different glands. Statistically significant changes are indicated by asterisks: * $P < 0.05$ and ** $P < 0.01$.

decreased transiently by approx. 0.05 pH units and then showed a sustained alkalinization of 0.09 ± 0.02 pH units ($n = 8$; averaged over the period 10–30 min following the onset of stimulation). Compared with the changes in phosphorus metabolites, the initial drop in pH_i was relatively rapid, and it is likely that with a time resolution of 2.5 min, the magnitude of the transient acidosis was underestimated. During infusion with supramaximal doses of acetylcholine (10^{-5} and 10^{-4} M), the changes in pH_i were similar to those caused by 10^{-6} M acetylcholine. Recovery of pH_i following withdrawal of acetylcholine was very slow and was only complete after about 20 min. It is also noticeable that the initial acidosis and subsequent alkalosis were not detected upon stimulation with 10^{-7} M acetylcholine, the concentration which evokes a half-maximal secretory response.

The chemical shift of the β-phosphate resonance of ATP in the unstimulated gland was -16.17 ± 0.02 ppm ($n = 17$). This corresponds to a ratio of $[MgATP]/[ATP]_t$ of $90.0 \pm 0.5\%$. Statistically significant increases in $[MgATP]/[ATP]_t$

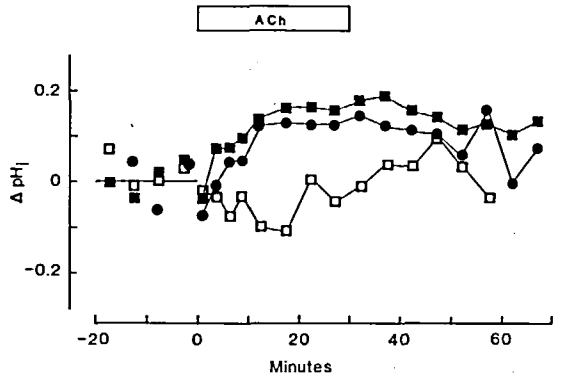

Fig. 7. Time course of changes in intracellular pH (ΔpH_i) in the perfused rabbit mandibular gland at 37°C on stimulation with three different concentrations of acetylcholine (ACh): 10^{-4} M (■), 10^{-6} M (●) and 10^{-7} M (□). Values were obtained from summed spectra from four to six different glands and are expressed as the difference from the mean resting value during the 20-min pre-stimulation control period in each group of experiments.

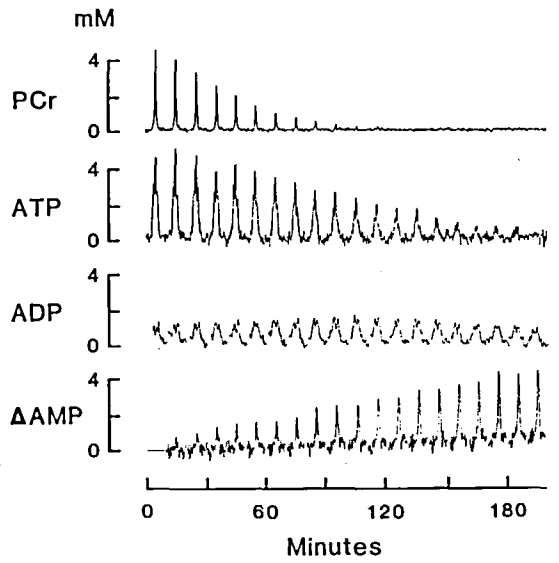

Fig. 8. Consumption of exogenous PCr and ATP induced by an homogenate of the rabbit mandibular gland at 25° C. The homogenate was added to a solution containing 5 mM PCr, 5 mM ATP, 5 mM $MgCl_2$ and 100 mM Hepes/Na^+-Hepes (pH 7.2). From the chemical shift of βATP, $[MgATP]/[ATP]_t$ was 98% under these conditions. Changes in the content of PCr, ATP (βATP, −15.9 ppm), ADP (βADP, −3.1 ppm) and AMP (6.5 ppm) were observed every 10 min. The line broadening factor was 2 Hz. Since the AMP resonance could not be distinguished from the phosphomonoester resonance of the homogenate, the change in AMP is shown as the difference spectrum after subtraction of the spectrum obtained in the first 10-min period.

were observed with 10^{-4} and 10^{-6} M acetylcholine. The changes, however, were very small (only 2–3%) when expressed as a fraction of the control value.

Discussion

As shown in Fig. 1, supramaximal concentrations of acetylcholine (10^{-5} and 10^{-4} M) elicit a reduced secretory flow during the plateau phase of secretion in the perfused rabbit mandibular gland compared with the response to the optimal concentration (10^{-6} M). This appears to be due to an enhancement of the phenomenon of tachyphylaxis or desensitization. In this study we have systematically investigated three possible side-effects of the accelerated cellular metabolism which accompanies secretion, any of which might contribute to the apparent inhibition of fluid transport: (1) exhaustion of the high-energy phosphate pool, (2) inhibition of metabolism due to intracellular acidification as a result of anaerobic metabolism, and (3) inhibition of ATP usage caused by a decrease in the availability of MgATP.

First, we can exclude the exhaustion of high-energy phosphates as an explanation. With the higher concentrations of acetylcholine (10^{-5} and 10^{-4} M), the decreases observed in ATP and PCr, and the increase in P_i, were similar to those induced by 10^{-6} M acetylcholine. Both the content of ATP and the total high-energy phosphate pool were maintained at approx. 65% of their respective control values during continuous stimulation.

With 10^{-4} M acetylcholine, significant increases in the content of AMP and ADP were observed. The increase in the size of the phosphomonoester/AMP resonance was attributed to AMP for the following reasons. (1) The chemical shift of the resonance in the difference spectrum after subtraction of the control spectrum (Fig. 5) was 6.5 ppm, which coincides with the chemical shift of AMP. (2) The increase in ADP was significantly smaller than the decrease in ATP (Fig. 3) which suggests that AMP was produced. (3) In an experiment in which an homogenate of the gland was added to a solution containing ATP and PCr, AMP appeared at the expense of ATP with little change in the content of ADP (Fig. 8). This indicates that adenylate kinase activity ($2ADP \rightleftharpoons ATP + AMP$) was present in the homogenate.

Since AMP would be expected to be slowly degraded to inosine, we cannot exclude the possibility that part of the AMP resonance in the homogenate experiment was due to IMP, which has the same chemical shift as AMP. However, the rate of synthesis of IMP under these conditions would be expected to be very slow (Takami, H., personal communication). Furthermore, in the perfused gland, the rapid decline in phosphomonoester/AMP content following the withdrawal of acetylcholine (Fig. 5) argues against any significant contribution from IMP.

Since the enzymes of glycolysis may be inactivated by low intracellular pH [12,13], it could be postulated that at the higher acetylcholine concentrations, the glycolytic pathway was inhibited

by an intracellular acidosis resulting from anaerobic metabolism. However, during the plateau phase of secretion, intracellular pH showed an alkalosis with 10^{-6} to 10^{-4} M acetylcholine (Figs. 6(a) and 7). This does not, however, indicate the absence of anaerobic metabolism in the gland. On the contrary, cessation of perfusion caused a large and rapid acidosis (data not shown). Furthermore, the marked increase in AMP during stimulation with 10^{-4} M acetylcholine suggests that there is an acceleration of the glycolytic pathway at this concentration [14]. It must be concluded, therefore, either that lactic acid produced by anaerobic glycolysis is released rapidly from the cells or that cellular pH regulatory mechanisms, such as the Na^+/H^+ antiporter, compensate for the acid load.

Since MgATP is the substrate for most cellular processes which nominally involve ATP [15], the parameter of importance in the assessment of ATP availability is the concentration of MgATP. Changes in this parameter can be determined from the chemical shift of the β-phosphate of ATP which is sensitive to changes in the ratio of MgATP to total ATP: it decreases, with reference to creatine phosphate, as $[MgATP]/[ATP]_t$ decreases. In this study, only a minimal change was observed in the chemical shift of the β-phosphate resonance of ATP on supramaximal stimulation with 10^{-4} M acetylcholine. The change indicated an increase in $[MgATP]/[ATP]_t$ of 3% (Fig. 6(b)) from a control value of 90%, which could be due to the accompanying alkalosis.

In conclusion, these results indicate that, as possible causes of the reduced secretory flow with supramaximal acetylcholine concentrations, we can exclude metabolic exhaustion, inhibition of metabolism due to intracellular acidosis and inhibition of ATP usage due to decreased MgATP availability.

Acknowledgements

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