Effects of Na⁺ Depletion on Fluid Secretion and Levels of Phosphorus Compounds as Measured by ³¹P-NMR in Perfused Canine Mandibular Gland

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Abstract The dependency of fluid secretion on extracellular Na⁺ and the levels of phosphorus compounds were studied in the perfused canine mandibular gland (using ³¹P-NMR). During control perfusion, the resting levels of creatine phosphate (CP) and ATP were 0.62 ± 0.05 mmol·kg⁻¹ gland and 0.42 ± 0.04 mmol·kg⁻¹ (mean $\pm$ S.E., $n=9$), respectively. Acetylcholine (Ach; 1 μ mol·l⁻¹ for 3 min) induced a salivary secretion and decreased the CP level. When Na⁺ in the perfusate was completely replaced with Li⁺, Ach induced only a minimal salivary secretion and no change in the ATP and CP levels. Restitution of Na⁺ to the perfusion, even without added Ach, caused a decrease in ATP and CP, and a small increase in salivary secretion. These results suggest that the activity of Na⁺/K⁺ ATPase is increased inversely via a rise of the intracellular Na⁺ concentration and that the salivary secretion is induced not only by added secretagogues but by an increase in the Na⁺ entry without added secretagogues.

Key Words: ³¹P-NMR, salivary gland, epithelial transport, ATP, creatine phosphate.

Fluid transport across the salivary gland and many other transporting epithelia depend on the extracellular Na⁺ concentration (CASE and HUNTER, 1982). It is believed that the Na⁺/K⁺ ATPase on the basolateral membrane of epithelial cells maintains a Na⁺ gradient across the membranes and the gradient can be utilized for Na⁺/Cl⁻ cotransport and thus secretion (POULSEN *et al.*, 1982; POULSEN and KRISTENSEN, 1982; YOUNG, 1982). If the Na⁺ gradient across the basolateral membrane is decreased by depletion of the extracellular Na⁺, the Na⁺ entry will

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be decreased. Decrease in Na^+ entry inactivates the Na^+/K^+ ATPase whose activity is controlled by intracellular Na^+ level and which maintains the Na^+ gradient. The inactivation of Na^+/K^+ ATPase may change levels of ATP and creatine phosphate.

In general, a quick and skillful chemical analytic technique is required to measure ATP and creatine phosphate of the organs whose energy metabolism is very high, such as the brain and salivary gland. Recently, the phosphorus magnetic resonance (^{31}P -NMR) has enabled us to non-invasively measure phosphorus compounds such as ATP, creatine phosphate, and tissue pH at the organ level. The ^{31}P -NMR has been used for measurements of phosphorus compounds in the perfused organs, such as rat heart (HOLLIS *et al.*, 1978), rat liver (McLAUGHLIN *et al.*, 1979), and canine salivary gland (MURAKAMI *et al.*, 1983). With this method, it is possible to measure the change in phosphorus compounds of the same gland along an experimental time course and test the reversibility of the phenomenon. The present study was undertaken on the perfused canine mandibular gland, using ^{31}P -NMR for measurement of phosphorus compounds during Na^+ -depletion, in order to study the relationship between fluid secretion and the cellular utilization of ATP.

METHODS

Dogs were anesthetized by intravenous injection of sodium thiopentothal ($0.04 \text{ g} \cdot \text{kg}^{-1}$ body weight). The mandibular gland was excised and perfused arterially at $10 \text{ ml} \cdot \text{min}^{-1}$ with an artificial perfusate in NMR tube (diameter 30 mm), at 25°C , as described previously (MURAKAMI *et al.*, 1983). The response to acetylcholine (Ach; $1 \mu\text{mol} \cdot \text{l}^{-1}$ for 3 min) was studied using control perfusate ($\text{Na}^+ = 151.0$, $\text{K}^+ = 4.3$, $\text{Mg}^{2+} = 1.0$, $\text{Ca}^{2+} = 2.5$, $\text{Cl}^- = 136.3$, $\text{HCO}_3^- = 25.0$, $\text{H}_2\text{PO}_4^- = 1.0$, glucose = $5.0 \text{ mmol} \cdot \text{l}^{-1}$ and 3% polyvinyl-pyrrolidone (M.W. = 40,000); gassed with 95% O_2 and 5% CO_2) and a Na^+ -depleted perfusate (Li^+ replacement). The salivary duct was cannulated for the secretory rate to be monitored by an electronic balance outside the NMR magnet (MURAKAMI *et al.*, 1980).

^{31}P -NMR studies were carried out using a Bruker WM-360wb NMR spectrometer operating at 145.8 MHz. The field frequency was controlled by locking the deuterium signal of D_2O in the glass tube (3 mm diameter). Radiofrequency pulses of $20 \mu\text{sec}$ were used as a 15° pulse. Free induction decays of 2,000 data points and sweep width of 10,000 Hz were used with an acquisition time of 0.12 sec, and 1,750 times accumulation was performed for 3.75 min. Using the same experimental protocol, NMR measurements were performed in four glands. These four groups of data were accumulated on a disc and transformed by a Fast Fourier Transform method (FFT). Accordingly, the transfers were plotted as averaged spectra for four glands. Resonance assignments were made from their chemical shifts, coupling constants and comparison with authentic samples, which agreed

Fig. 1. The effect of Na^+ depletion on salivary secretory rate of an isolated perfused canine mandibular gland. Typical changes in the salivary secretory rates ($\mu\text{l} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ weight) and the volume of accumulated saliva (** $\text{ml} \cdot \text{g}^{-1}$ tissue weight) of the same gland were recorded during control (panel a: KHR, Krebs-Henseleit Ringer solution: A, acetylcholine $1 \mu\text{mol} \cdot \text{l}^{-1}$ for 3 min), during Na^+ -depletion (panel b: LiR, Na^+ -depleted Li $^+$ Ringer solution), during Na^+ restitution (panel c), and during recovered control perfusion (panel d).

with assignments reported by others (BURT *et al.*, 1976; HOULT *et al.*, 1974; NAVON *et al.*, 1977). Tissue levels of phosphorus metabolites were determined as the areas underneath the corresponding resonance peaks in the spectrum. The peak area of the resting control gland was calibrated by comparison with a measured peak area of the standard solution in a reservoir of the same volume as a used gland.

RESULTS

Fluid secretion

During the control perfusion, an acetylcholine administration (Ach; $1 \mu\text{mol} \cdot \text{l}^{-1}$ for 3 min) induced a salivary secretion. The maximum rate of secretion was $125.5 \pm 10.9 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ (mean $\pm$ S.E., $n=10$) and the secretion lasted for 10–15 min. Figure 1-a shows a typical example of the fluid secretion by Ach during control perfusion. During the Na^+ -depleted perfusion, no secretion was induced unless Ach was added. During Na^+ depleted perfusion, the first administration of Ach induced a small secretion. The maximum rate of secretion was $27.6 \pm 6.5 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ($\pm$ S.E., $n=8$) and this secretion lasted for 6–8 min (Fig. 1-b). But the second administration of Ach did not induce any secretion. About 10 min after the restitution of Na^+ to the perfusion, a small amount of saliva began to be secreted without any added secretagogues. The maximum rate of secretion

Fig. 2. The effect of Na^+ depletion on ^{31}P -NMR spectrum in the isolated perfused canine mandibular glands. Typical ^{31}P -NMR spectrum from groups of four glands. Sequential 3.5-min spectra were collected along the experimental time course. Spectrum a: resting state during control perfusion. Spectrum b and c: ACh ($1 \mu\text{mol}\cdot\text{l}^{-1}$) stimulation during control perfusion. Spectrum d and e: ACh stimulation during Na^+ depletion. Spectrum f: Na^+ restitution. Peaks are: sugar phosphates and AMP (1), inorganic phosphate (2), creatine phosphate (3), γ -ATP and β -ADP (4), α -ATP, α -ADP, NAD^+ and NADH (5), and β -ATP (6).

Fig. 3. Time course of changes in tissue levels of creatine phosphate by Ach ($1 \mu\text{mol} \cdot \text{l}^{-1}$, for 3 min) during control and Na^+ -depleted perfusion. Tissue level of creatine phosphate was estimated from areas underneath the corresponding resonance peaks in the spectrum and expressed by the percentage of resting level during control perfusion. KHR, A, and LiR are the same as in Fig. 1.

was $5.1 \pm 1.7 \mu\text{l} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ($\pm \text{S.E.}$, $n=8$) and this secretion lasted for 7–10 min (Fig. 1-c). After this secretion, the same gland responded inversely to Ach stimulation and secreted as much as the control level (Fig. 1-d).

Creatine phosphate (CP)

During the control perfusion, the resting level of creatine phosphate (CP) was $0.62 \pm 0.05 \text{ mmol} \cdot \text{kg}^{-1}$ tissue weight (mean $\pm \text{S.E.}$, $n=9$). The resonance of creatine phosphate (CP) is shown as peak 3 in Fig. 2-a. Acetylcholine (Ach) administration decreased the CP to 30% of the control level (Figs. 2-b, c and 3). About 20 (≈ 10.5 – 31.5) min after the end of the stimulation, the CP recovered to the normal level. The Na^+ -depletion from the perfusion did not change the CP level. During the Na^+ -depletion, even added Ach did not change the CP level (Figs. 2-d, e and 3). The Na^+ -depletion continued for about 40 min, thereafter Na^+ was restituted to the perfusion. The Na^+ -restitution decreased the CP level. Then the CP level was decreased to 60% of the control level (Fig. 3). The rate of decrease in CP by Na^+ -restitution was slower than that by Ach during control perfusion. About 30 min after the start of the Na^+ -restitution, the CP level returned to the control level (Fig. 3). Thereafter, Ach administration decreased the CP level again in the same gland. After Ach stimulation, the CP level recovered to near the control level.

Figure 3 shows the time plot of CP level as a percentage of the resting level during the control perfusion. Data were calculated from the areas of resonance depicted in Fig. 2, in which the spectra were plotted as an averaged resonance of four glands, as described in METHODS.

Fig. 4. Time course of changes in tissue levels of ATP by Ach ($1 \mu\text{mol}\cdot\text{l}^{-1}$, for 3 min) during control and Na^+ -depleted perfusion. Tissue levels of ATP were estimated from the areas underneath the corresponding resonance peaks in the spectrum and expressed by the percentage of the resting level during control perfusion. KHR, A, and LiR are the same as in Fig. 1.

ATP

During the control perfusion, the resting level of ATP was $0.42 \pm 0.04 \text{ mmol}\cdot\text{kg}^{-1}$ tissue weight ($n=9$, $\pm\text{S.E.}$). The resonance of ATP is shown as peak 6 in Fig. 2-a and identified as β -ATP. Ach stimulation did not change the ATP level during control perfusion (Figs. 2-b, c, and 4). When oxygen supply to the gland is inadequate due to an arterial embolism or a venous occlusion, an added Ach sometimes decreases the ATP level during control perfusion (MURAKAMI *et al.*, 1983). In the present study, Ach did not decrease ATP level, suggesting that oxygen supply to the gland was adequate for maintaining ATP level in the present perfused gland.

The depletion of Na^+ from the perfusion did not change the ATP level either. During the Na^+ -depletion, the Ach administration did not change the ATP level of the gland (Figs. 2-d, e, and 4). The Na^+ restitution decreased the ATP level slowly to 80% of the control level (Figs. 2-f and 4).

Figure 4 shows the time plot of ATP level as a percentage of the resting level during the control perfusion. Data were calculated from the resonance areas depicted in Fig. 2, in which the spectra were plotted as an averaged resonance of four glands, as described in METHODS.

DISCUSSION

^{31}P -NMR in the isolated perfused organs enabled us to simultaneously measure fluid transport and levels of phosphorus energy metabolites (ATP, ADP, creatine phosphate, sugar phosphate, NAD, NADH, inorganic phosphate *etc.*) along the

experimental time course under different conditions of vascular perfusion of the organs (HOLLIS *et al.*, 1978; McLAUGHLIN *et al.*, 1979; MURAKAMI *et al.*, 1983). Vascular perfusion is the best way to supply oxygen to tissue. This method allows maintenance of the integrated functions which are related to the anatomical structure of tissue, such as glandular secretion and ductal reabsorption. Furthermore, vascular perfusion does not destroy the anatomical structure for oxygen supply, such as the capillary bed (RENKIN, 1962).

But there are several problems in using NMR for the perfused organs. One of them is homogeneity of the material. The perfused organ set in the tube is very heterogeneous, because of the structure of the organ itself and the tubes added for perfusion, suction, and sampling. In the case of perfused organ, one cannot use a spinning of the NMR tube for homogeneity of the materials, because of the torsions of perfusion tubes. Accordingly, it is necessary to unify the magnetic field in the NMR magnet by a precise modulation in the electrical circuit (called "shim") each time after setting the sample tube in the NMR magnet. Another problem is the determination of absolute amounts of phosphorus compounds. There are some techniques for calibration. The most reliable method is to compare the resonance area of standard NMR spectrum measured at the end of the experiment with the amount measured by the chemical analysis of the same material which is excised and frozen immediately after the experiment. An alternative method is based on the measurement of a standard substance, for example, an inorganic phosphate in the perfusate or in the model reservoir whose volume is the same as that of the material used. In the present study we used the latter method, which proved a simple and quick method, but still required comparison with the amounts measured by chemical analysis. The estimated amounts of CP and ATP were comparable to the reported values measured by chemical analysis (NORTHUP, 1935; DREISEBACH and GERLACH, 1967).

A change in tissue levels of creatine phosphate (CP) and ATP reflects an imbalance in the "supply" and "consumption" of the common energy source, ATP. As already described (MURAKAMI *et al.*, 1983), the cessation of the perfusion caused hypoxia of the tissue and reduced the "supply" of ATP from the oxidative metabolism. The level of CP decreased more quickly than the level of ATP, suggesting that CP is a quick ATP-donner *via* a Lohmann reaction and contributes largely to the energy metabolism in the salivary gland.

The secretory stimulation increased oxygen consumption in the salivary gland (TERROUX *et al.*, 1959; MURAKAMI, 1981), indicating that the "supply" of ATP from the oxidative metabolism is increased during the salivary secretion. The present results indicate that acetylcholine (Ach) caused no change in the ATP level but decreased the level of CP (Figs. 2-b, c, 3, and 4). These findings suggest that an increase in the "supply" of ATP is mainly *via* a Lohmann reaction and compensates the increase in the "consumption" of ATP. The possibility that Ach indirectly increased the "consumption" of ATP is supported by the reports

that Ach increased the Na^+ entry in the salivary gland (IMAI, 1965; BURGEN, 1956; POULSEN, 1974; GOTO, 1981). This is because an increase in the Na^+ entry could increase the activity of the Na^+/K^+ ATPase, thus increasing the "consumption" of ATP.

During the Na^+ -depleted perfusion, an added Ach did not decrease the levels of ATP and CP (Fig. 2-d, e). These findings suggest that the activity of Na^+/K^+ ATPase is low during the Na^+ -depleted perfusion. The Na^+ gradient across the basolateral membrane plays an important role in the fluid secretion and the activation of Na^+/K^+ ATPase. The Na^+ depletion decreases the Na^+ gradient, which could reduce Na^+ entry. The reduced Na^+ entry decreases intracellular Na^+ and the lowered Na^+ level reduces the activity of the Na^+/K^+ ATPase. Accordingly, the intracellular K^+ could possibly leak out from cells during the Na^+ depletion.

In addition, the "supply" of ATP from the oxidative metabolism is possibly decreased during the Na^+ -depleted perfusion. This possibility is supported by reports that the rate of oxygen consumption decreased during Na^+ depletion in several epithelial tissues, such as toad skin (DANISI and VIEIRA, 1974), rabbit gall bladder (MARTIN and DIAMOND, 1966), bullfrog cornea (REINACH *et al.*, 1977), and stimulated rat salivary gland (SAKAMOTO *et al.*, 1981).

At the Na^+ restitution, the levels of ATP and CP were decreased (Figs. 2-f, 3, and 4). These findings indicate that the "consumption" of ATP was beyond the "supply" of ATP at the Na^+ restitution, that is, an increased consumption and/or a decreased supply of ATP. A decreased supply of ATP at the Na^+ restitution is possibly due to the slow recovery of oxidative metabolism suppressed during Na^+ depletion as mentioned above. The late recoveries of the CP and ATP levels (Figs. 3 and 4) suggest this slow recovery of the oxydative metabolism. An increased consumption of ATP by the Na^+/K^+ ATPase is possibly due to an increase in the Na^+ entry. One possible explanation is that the Na^+ gradient is increased by the Na^+ restitution. Na^+ can leak out during the Na^+ depletion and the intracellular Na^+ level is lowered. Accordingly, when an extracellular Na^+ is increased by the Na^+ restitution, the Na^+ gradient for Na^+ entry will be increased more than the Na^+ gradient during the control perfusion. A second possibility is that a hyperpolarization is caused by the Na^+ restitution, increasing the Na^+ electrochemical potential. The intracellular K^+ can be decreased during the Na^+ depletion, as mentioned above. As extracellular K^+ level is constant in the present study, a membrane potential could be hyperpolarized during the Na^+ depletion and at the initial phase of the Na^+ restitution. In addition, when the activity of the Na^+/K^+ ATPase was increased, the membrane potential could be hyperpolarized because Na^+/K^+ ATPase is electrogenic. The third possibility is that the permeability for Na^+ could be increased by Na^+ restitution. The Na^+ restitution could possibly stimulate some receptors on cells or the intraglandular nerve endings to release acetylcholine and/or adrenaline. In the present study,

neither atropine nor any other adrenergic blockers were used, thus this possibility remains to be investigated. The fourth possibility for Na^+ entry is that the H^+ site of Na^+/H^+ antiporter on the vesicle of the renal brush border is replaceable by Li^+ (KINSELLA and ARONSON, 1981). At the Na^+ restitution, an increased Li^+ efflux could couple with the Na^+ entry. But in the present study the intracellular Na^+ level was not measured, so how much the Na^+ was increased at the Na^+ restitution is unknown.

A small salivation was observed on the Na^+ restitution (Fig. 1). This finding suggests that the gland can secrete saliva only with Na^+ entry without added Ach. As described above, there are several possibilities for the increased Na^+ entry at the Na^+ restitution.

In the present study, Na^+ in the perfusate was completely replaced by Li^+ . Li^+ replacement experiments have previously been performed on the perfused rat mandibular gland (COMPTON *et al.*, 1981). It is unclear whether or not Li^+ can enter the cell in the salivary gland. In Li^+ therapy, the saliva to plasma Li^+ ratio in final saliva is 2 to 3 (GROTH *et al.*, 1974). Li^+ can be reabsorbed by the submandibular excretory duct of rat (UEHA and SCHNEIDER, 1970). The Na^+/H^+ antiporter of the renal brush border was reported to have a high affinity for Li^+ (KINSELLA and ARONSON, 1981). These findings suggest that Li^+ can enter the salivary cells.

It is widely accepted that Ach increases the permeability of cations such as Na^+ and K^+ in the salivary acinar cells. It has recently been proposed that permeability of non-specific cation channels is increased by Ach (PETERSEN and MARUYAMA, 1983). There is a possibility that Li^+ entry could be increased *via* this non-specific cation channel by Ach during the Na^+ free Li^+ perfusion.

The Na^+ site of the Na^+/K^+ ATPase has no affinity to Li^+ in many epithelial tissues (EHRICH and DIAMOND, 1980). As mentioned above, Li^+ can enter the cell. But, in the present study, the CP and ATP levels were not decreased by Ach during the Li^+ perfusion. This finding supports the theory that the Na^+ site of the Na^+/K^+ ATPase of the salivary gland has no affinity to Li^+ , as in many other epithelial tissues.

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