

## EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES UPON INTRACELLULAR SODIUM IN RAT SKELETAL MUSCLE

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### 1. Introduction

In vitro addition of insulin stimulates not only active  $\text{Na}^+$  efflux from frog sartorius [1], but also  $(\text{Na}^+ + \text{K}^+)$ -ATPase activity in plasma membrane fragments isolated from frog skeletal muscle [2]. Based upon these findings, Moore [1] and Gavryck et al. [2] proposed that stimulation of the  $\text{Na}^+$  pump by insulin plays a physiological role in the action of this hormone. Although the concentration (250 mU/ml) of insulin used in these experiments was comparatively high, it is about the same concentration required, when albumin is not used, to increase oxygen consumption [3,4], lactate production [5], 3-O-methyl-D-glucose transport [6], and the plasma membrane potential [7] of frog sartorius muscle.

The requirement for insulin concentrations  $\sim 10^4$ -times the physiological levels ( $\sim 30 \mu\text{U}/\text{ml}$  for rats [8]) may represent species difference and/or adsorption of insulin to glassware [9]. This is supported by the finding that a concentration of insulin well within the pathophysiological range ( $350 \mu\text{U}/\text{ml}$ ) produced a very significant increase in renal tubular reabsorption of  $\text{Na}^+$  in the isolated dog kidney [10]. Moreover, addition of albumin to the incubation medium lowers the concentration of insulin required to stimulate active  $\text{Na}^+$  efflux from rat soleus muscle from 100 mU/ml to  $100 \mu\text{U}/\text{ml}$  [11].

Since streptozotocin diabetes is accompanied by a decrease in plasma insulin levels [8], the hypothesis that physiological levels of insulin regulate the sodium pump is tested in the present study by inducing streptozotocin diabetes in rats and comparing the levels of intracellular  $\text{Na}^+$  ( $\text{Na}_i^+$ ) in muscle

with those in muscles from control animals.  $\text{Na}_i^+$  of soleus muscle was found to be significantly elevated in 28 of 39 rats made diabetic by streptozotocin injection, and all of the 23 animals with plasma glucose  $> 300 \text{ mg}/100 \text{ ml}$  had significantly elevated  $\text{Na}_i^+$ .

### 2. Materials and methods

#### 2.1. Induction of diabetes and sample preparation

Wistar rats of both sexes (160–320 g body wt) bred in our animal facility were used in all experiments. The diabetic state was induced by a single intraperitoneal injection of streptozotocin (Sigma) at a dose of 75 mg/kg body wt. The streptozotocin was prepared in acidified (pH 4.5) citrate buffered saline immediately prior to injection. Rats were provided with a 5% glucose solution to drink during the first 48 h post-injection in order to insure survival through the hyperinsulinemic period resulting from streptozotocin-induced beta cell lysis.

Efforts were taken to minimize stress to the animals prior to sacrifice, which occurred from 2–28 days after injection. After the rat was rendered unconscious by a sharp blow to the cranium, blood (2–4 ml) was immediately withdrawn by cardiac puncture into a heparinized syringe (Li heparin, Sigma) and transferred into a heparinized centrifuge tube. Plasma was separated from cells by centrifugation and stored in glass tubes at  $-20^\circ\text{C}$  until use. Before freezing, an aliquot of plasma was diluted with deionized water for determination of plasma sodium by flame emission photometry. The plasma glucose concentrations were

determined by Glucostrate (a gift of General Diagnostics), a colorimetric assay specific for glucose and glucose-6-phosphate utilizing hexokinase and glucose-6-phosphate dehydrogenase.

## 2.2. Washout of $\text{Na}^+$ from extracellular space

Immediately after cardiac puncture, both hind limbs were removed and paired soleus muscles (50–70 mg) were dissected; this required a maximum of 5 min. Muscles used for analysis of  $\text{Na}^+$  content were mounted at rest length on platinum frames. All studies upon isolated muscles were conducted at  $21 \pm 1^\circ\text{C}$ .

Muscles used for radioactive sodium studies were loaded with  $^{22}\text{Na}$  by immersion for 90 min in 1 ml oxygenated Ringer containing 60  $\mu\text{Ci/ml}$   $^{22}\text{Na}$ , 140 mM NaCl, 2 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{NaH}_2\text{PO}_4$ , 0.8 mM  $\text{Na}_2\text{HPO}_4$ , and 1 mM ouabain. The muscles were then transferred through a series of 14 washout tubes containing 8 ml Na-free and K-free Ringer composed of 98 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{CaCl}_2$ , 1.4 mM  $\text{MgSO}_4$ , 1.6 mM  $\text{MgHPO}_4$ , and 1 mM ouabain. The time intervals between transfers were selected in order to accurately follow the washout curve as in fig. 1a, where the  $^{22}\text{Na}$  in the muscle was determined by back-addition of the counts per minute (cpm) in the washout tubes as per [1]. Both the  $^{22}\text{Na}$  loading of the muscles and their subsequent washout were conducted in a 100%  $\text{O}_2$  atmosphere to insure continued tissue viability. After washout, the muscles were removed from their frames, blotted on filter paper, weighed, and dried at  $110^\circ\text{C}$  overnight to determine wet and dry weights [1]. Each washout tube (and the muscle) was counted in an automatic well-type solid scintillation counter for a total of 60 000 counts.

Muscles were prepared for flame emission analysis of  $^{23}\text{Na}$  by ashing at  $850^\circ\text{C}$  for 14 h and dissolving the ash in 0.1 N  $\text{HNO}_3$  for 30 min. In the  $^{22}\text{Na}$  washout experiments, each washout tube was also analyzed for  $^{23}\text{Na}$  by flame emission photometry.

All results are given as the mean  $\pm$  the standard error.

## 3. Results

### 3.1. Intracellular $\text{Na}^+$ determination

Determination of intracellular sodium is com-

pllicated by the presence of substantial amounts of  $\text{Na}^+$  in the extracellular space (ECS). Using an average value of ECS from the literature to estimate extracellular  $\text{Na}^+$  is unreliable since the relative size of ECS varies with the size of the muscle in rats [12] by  $\leq 3$ -fold. In the present experiments, ECS varies from  $\sim 20$ –35% of wet weight of muscles of approximately the same size.

In frog sartorius, the problem of correcting for ECS can be avoided altogether by equilibrating all  $\text{Na}^+$  within the muscle, effluxing, plotting the logarithm of back-added cpm and extrapolating the slow (cytoplasmic) component to time zero [7,13] to give  $\text{Na}_i^+$ . Neville [14] analyzes the washout curves of  $^{22}\text{Na}$  from frog sartorius into a sum of three exponential components, and shows that two components represent washout from ECS while the third (slowest) component is due to intracellular  $\text{Na}^+$ . Because of the importance of accurate estimates of  $\text{Na}_i^+$ , the applicability of this analysis to rat soleus was first determined. Figure 1a shows a typical washout curve

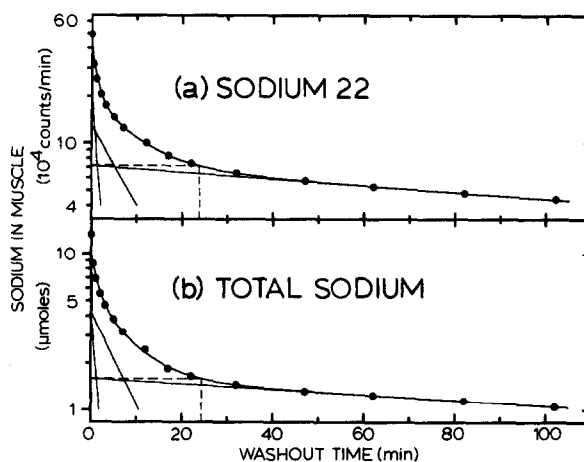


Fig. 1. (a) Washout of radioactive sodium from a rat soleus muscle loaded with  $^{22}\text{Na}$ . (b) Washout of total sodium from the same muscle, as determined by flame emission spectrophotometry. In each case, the data is separated by a curve peel technique into three exponential components, which are represented by straight lines on the semilogarithmic plot. The curve is the sum of these three components and provides a good fit to the experimental data. The  $t=0$  intercept of the slowest component is taken as an estimate of intracellular sodium; and the dotted line yields the time at which the sodium remaining in the muscle, as given by the computed curve, equals this estimate of intracellular sodium.

of  $^{22}\text{Na}$  from a 56 mg rat soleus muscle in the presence of ouabain. As in frog skeletal muscle, the entire curve consists essentially of three exponential components, each of which is plotted separately in fig.1a.

Since it is impractical to load each rat muscle with  $^{22}\text{Na}$  in experiments of this type,  $\text{Na}^+$  content was determined using flame emission spectrophotometry for each of the efflux tubes in this experiment, and the results back added as with  $^{22}\text{Na}$ . Figure 1b shows the logarithm of total  $\text{Na}^+$  in the muscle, determined in this manner, versus time. The curve again consists essentially of a sum of three components with each component having a rate constant nearly identical to that determined using  $^{22}\text{Na}$ . This means that flame emission analysis of muscle washout can be used without radioactive  $\text{Na}^+$ . Moreover, the procedure can be 'calibrated' by taking that time at which the curve has the value of  $\text{Na}_i^+$  obtained by back extrapolation. As seen in fig.1, this time interval is essentially the same whether  $\text{Na}^+$  is determined analytically or by  $^{22}\text{Na}$ . In 7 such experiments, the interval averaged  $21.5 \pm 0.7$  min when determined analytically and  $22.2 \pm 0.6$  min when determined by  $^{22}\text{Na}$ . Therefore, one single measurement of muscle  $\text{Na}_i^+$ , done after 22 min of a washout in a  $\text{Na}$ -free medium containing ouabain would have provided a highly accurate estimate of  $\text{Na}_i^+$  in each of these experiments and presumably in any experiments where muscles of this size are treated in the same manner. Indeed, when estimating  $\text{Na}_i^+$  in each of these 7 muscles, the value given by the single point at 22 min differed from the back-extrapolated intercept by  $-2.8 \pm 2.0\%$  when determined by flame analysis, and by  $+2.5 \pm 1.1\%$  when determined on the  $^{22}\text{Na}$  curve.

### 3.2. Effect of streptozotocin diabetes upon intracellular $\text{Na}^+$

Accordingly, flame analysis of the muscle after 22 min of washout was used to estimate  $\text{Na}_i^+$  in muscles from streptozotocin-diabetic rats. These rats were sacrificed from 2–28 days after streptozotocin injection and muscle  $\text{Na}_i^+$  and plasma glucose were determined. The results from these 39 animals are illustrated in fig.2, where muscle  $\text{Na}_i^+$  is plotted versus plasma glucose for each animal. Of the 30 animals which were hyperglycemic, all but 7 had significantly ( $P < 0.05$  in each case) elevated  $\text{Na}_i^+$ , and

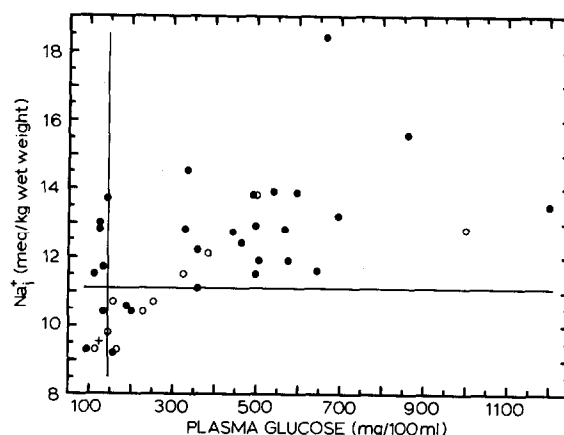


Fig.2. Plasma glucose and intracellular sodium in soleus muscle for each of the 39 streptozotocin-injected rats. Open circles represent animals sacrificed 2 or 4 days after injection; solid points represent animals sacrificed 7–28 days after injection. The cross denotes the mean values (129.5 mg glucose/100 ml, 9.52 mequiv.  $\text{Na}^+$ /kg muscle wet wt) for 15 control animals. The latter is consistent with the estimate of 8.9 mequiv.  $\text{Na}^+$ /kg in [11] for rat soleus muscle. The vertical (145 mg/100 ml) and horizontal (11.1 mequiv./kg wet wt) lines are the 95% upper limits for, respectively, plasma glucose and intracellular sodium in the controls.

of these, 4 were animals sacrificed only 2–4 days after injection with streptozotocin. Elevated  $\text{Na}_i^+$  was found in soleus muscles from animals sampled during the entire 28 day experimental period. All 23 animals with plasma glucose  $> 300$  mg/100 ml had significantly elevated  $\text{Na}_i^+$ . Only 4 animals had normal levels of both  $\text{Na}_i^+$  and plasma glucose; of these, 2 were sacrificed within 4 days of injection. Of special interest is the fact that of the 39 animals (all of which exhibited clinical symptoms of diabetes such as a decreased rate of weight gain), 5 had a statistically significant increase in muscle  $\text{Na}_i^+$ , notwithstanding normal levels of plasma glucose.

It might be argued that the increase in  $\text{Na}_i^+$  was caused by hypernatremia due to possible dehydration secondary to polyuria. However, the mean plasma  $\text{Na}^+$  concentration of controls,  $142.9 \pm 8.0$  mM, was not significantly different from the mean,  $142.4 \pm 7.9$  mM, of the 39 streptozotocin-injected animals. Indeed, only one of these 39 had plasma  $\text{Na}^+$  greater than the 95% confidence interval, whereas 28 of the 39 had significant elevations of  $\text{Na}_i^+$ .

#### 4. Discussion

Streptozotocin diabetes is accompanied by a decrease in plasma insulin levels [8]. The present study demonstrates that this is associated with a significant elevation in muscle  $\text{Na}_i^+$  in 28 (72%) of 39 animals. This supports the hypothesis that physiological levels of insulin regulate intracellular  $\text{Na}^+$ , consistent with the suggestion that insulin plays a physiological role in the regulation of the  $\text{Na}^+$  pump.

It might be argued that streptozotocin has a direct inhibitory action upon the sodium pump. However, since at least some cells have been shown to produce an increased number of sodium pumps within 24 h in response to elevated  $\text{Na}_i^+$  [15,16], such an effect would likely be limited to the first 2–4 days post-injection. In contrast, the results reported here demonstrate that 28 days post-injection  $\text{Na}_i^+$  is still elevated. This is consistent with the hypothesis that a decrease in plasma insulin results in a decreased sodium pump activity.

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