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INSULIN ACTION

ACCUMULATION IN VITRO OF Mg^{2+} AND K^+ IN RAT UTERUS: ION PUMP ACTIVITY*

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SUMMARY

Surviving hemiuteri from donor ovariectomized rats were incubated in Krebs–Henseleit buffer, modified in select experiments with respect to Ca^{2+}, Mg^{2+} and/or K^+. These studies demonstrated the following:

1. The retrogressed uterus *in vitro* maintains a substantial concentration gradient, relative to the medium, for both Mg^{2+} (3.8:1 in 1.2 mM, 38:1 in 0.12 mM Mg^{2+}) and K^+ (13:1).

2. Insulin added *in vitro* promotes a net increase in the accumulation of Mg^{2+} and of K^+ (7–16%) in uterine muscle. Mg^{2+} influx into the tissue does not occur by diffusion *per se*, and it is in general influenced by the same factors as is K^+ accumulation. Under the conditions of incubation used, neither the Na^+ nor the Ca^{2+} content was affected by insulin.

3. Increases in Mg^{2+}, K^+ content (insulin effect) are not associated with alterations in the extracellular space (D-[1-^{3}H]sorbitol or Na^+ space).

4. Ouabain ($5 \cdot 10^{-4}$ M) added *in vitro* gives rise to very striking changes in this muscle: (a) Na^+ content increases; (b) K^+ content decreases, but less than the Na^+ increases; (c) Mg^{2+} content increases to levels as high as that obtained with insulin in the absence of ouabain; (d) no additional effect on Mg^{2+} accumulation can be shown when insulin is added to a medium that also contains ouabain.

5. Omission of K^+ from the incubation medium mimics the ouabain effect insofar as: (a) Na^+ content increases; (b) Mg^{2+} content increases; (c) no additional effect of insulin added *in vitro* is obtained.

6. The interaction of insulin with its receptor in the plasma membrane appears to engage transport systems, including the ion pump [($Na^+ + K^+$)-ATPase (ouabain sensitive; ATP phosphohydrolase, EC 3.6.1.3)]; how increased translocation of cations which influence enzymatic activities, *i.e.* Mg^{2+} and K^+, may lead to amplification of the insulin signal at the membrane is discussed.

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INTRODUCTION

The insulin stimulus to protein synthesis in isolated epididymal adipose tissue[1] and adipose cells[2] is dependent upon the presence of Mg^{2+} and Ca^{2+} in the incubation medium. This poses the possibility that an early event in insulin action involves the translocation of ions, and that translocation of critical ion(s) in turn is required to activate one or more components of the cellular synthetic machinery, including protein synthesis[3].

To explore this hypothesis we have determined the effect of insulin added *in vitro* on the Mg^{2+}, K^+, Na^+ and Ca^{2+} content of rat uterus. These analyses demonstrate that insulin does cause a net increase in the accumulation of Mg^{2+} and K^+ in retrogressed uterus, and that a functioning ion pump is one requisite for this action of insulin. From the latter results we deduce that the receptor for insulin located in the plasma membrane is coupled to (Na^+ + K^+)-ATPase (ouabain sensitive; ATP phosphohydrolase, EC 3.6.1.3), and that activation of the insulin receptor engages the ion pump.

MATERIALS AND METHODS

In vitro incubations

Retrogressed uteri were used in all experiments. Donor rats of the Holtzman strain were ovariectomized at 24 days of age; 7 weeks or longer after the operation, they were sacrificed by decapittation and the uteri removed, bisected at the cervix and incubated for 2 h (36 °C) in 2 ml of Krebs–Henseleit buffer[4], modified as indicated with respect to Mg^{2+}, Ca^{2+} or K^+, that contained either sodium pyruvate or glucose (10 mM). Pyruvate was used as substrate in most experiments, for 2 reasons: to separate insulin effects on ion transport from those on glucose transport, and to obviate possible insulin-related shifts in cell water that have been observed only when glucose is present in the medium[5]. One hemiuterus from each animal served as control, the other as test; the gas phase was CO_2–O_2 (5:95, v/v).

In two experiments to determine tissue extracellular space independent of Na^+ measurements (Table II), [^{3}H]-sorbitol, together with unlabeled sorbitol, was added to give a final concentration of 2.5 μCi (5 μmoles) per ml of medium. The procedure described by Crofford and Renold[6] for adipose tissue was followed.

Processing of uteri and ion analyses

Following incubation, hemiuteri were withdrawn from the incubation medium, rinsed quickly in 5 ml of isotonic sucrose (0.25 M), blotted on sucrose-dampened filter paper, and weighed on a torsion balance. The organs were then processed for analysis of ion content by one of three procedures, each of which yields unique data pertinent to the problems posed: dissolution in HNO_3 (Na^+, K^+ and Mg^{2+} content of individual hemiuteri; Ca^{2+} cannot be estimated directly by this procedure); or, incineration (Ca^{2+} in addition to K^+ and Mg^{2+} content; pooled samples of uteri required); or, trichloroacetic acid extraction (incorporation of labeled amino acid into protein, also K^+ and Mg^{2+} content; Na^+ determinations cannot be made using this procedure because of Na^+ contamination incurred during homogenization in Pyrex tubes).

Ion determinations were made, in duplicate, with a Unicam atomic absorption

spectrophotometer; all solutions used as standards were prepared exactly as the test samples. In analyses for Mg^{2+} and Ca^{2+}, dipotassium ethylenediaminetetracetic acid (EDTA) was added to give a final concentration of 0.03–0.04 M; following addition of EDTA samples were gently mixed and analyzed shortly thereafter in order to circumvent the formation of interfering precipitates at acid pH.

Materials

L-[2,5-$^3H_2$]Histidine (40 Ci/mmole) was a product of Amersham/Searle; it was diluted with unlabeled DL-histidine in saline to give a final concentration of 0.03 μmole /ml of incubation medium. D-[1-^{3}H]Sorbitol) (6.8 Ci/mmole) was purchased from New England Nuclear, Boston. Sodium pyruvate was obtained from Nutritional Biochemicals Corporation, Cleveland, ouabain (g-strophanthin) from Schwarz/Mann, Los Angeles. A sample of beef insulin of low glucagon content was kindly provided by Dr E. L. Grinnan of Eli Lilly and Company, Indianapolis; the hormone was prepared as a stable stock solution in 0.003 M HCl (1 mg/ml) and added to the incubation medium just prior to introduction of the test tissue.

RESULTS

Insulin stimulation of Mg^{2+} and K^+ accumulation in relation to the Ca^{2+} and Mg^{2+} concentrations of the medium (Table I)

TABLE I

EFFECT OF INSULIN ON Na^+, K^+ AND Mg^{2+} CONTENT OF RAT UTERUS

The substrate was 10 mM sodium pyruvate; tissues were analyzed after dissolution in HNO_3. Single hemiuteri were transferred to Na^+-free Pyrex tubes, capped, and dried overnight at 70 °C. 50 μl of concentrated HNO_3 were then added and the contents hydrolyzed at room temperature with intermittent mixing over 24 h. After dilution with ion-free water (to a total volume of 300 μl), 10-μl and 100-μl aliquots were taken for K^+–Na^+ and Mg^{2+} determinations, respectively. Number of hemiuteri used is shown in parentheses; mean values ± S.E., and probability of difference (P) from no insulin control being due to chance are given. U = control hemiuteri that were not incubated. Throughout the study uteri were incubated for a period of 2 h; no significant effect of insulin on Mg^{2+} or K^+ accumulation could be demonstrated when incubations were terminated after only 15, 30 or 60 min.

Ions in medium (mM/l)		*Insulin (0.1 unit/ml)*	*Wet weight (mg)*	*Ion concentrations found (μmoles/g wet wt)*		
Ca^{2+}	Mg^{2+}			Na^+	K^+	Mg^{2+}
1.27	1.20	− (10)	17.1 ± 0.9	57.0 ± 2.5	76.8 ± 1.3	4.49 ± 0.08
		+ (10)	16.1 ± 0.9	57.1 ± 1.7	84.0 ± 0.8	5.14 ± 0.12
					($P < 0.001$)	($P < 0.001$)
		U (8)	15.1 ± 0.2	62.3 ± 0.8	73.8 ± 0.7	4.61 ± 0.17
2.54	0.12	− (13)	17.6 ± 0.9		74.2 ± 2.0	4.49 ± 0.12
		+ (13)	16.7 ± 0.8		82.5 ± 1.0	5.02 ± 0.12
					($P = 0.001$)	($P < 0.007$)
		U (10)	18.1 ± 0.6		70.8 ± 1.1	4.65 ± 0.08
1.27	0.12	− (8)	16.6 ± 0.5		71.0 ± 1.2	4.73 ± 0.08
		+ (8)	16.5 ± 0.5		75.6 ± 0.2	5.06 ± 0.08
					($P < 0.003$)	($P < 0.01$)
		U (8)	17.0 ± 0.5		66.9 ± 1.3	4.36 ± 0.04

Incubations were carried out in Krebs–Henseleit bicarbonate[4] as the basic medium. In this buffer, which has an ionic composition approximating that in plasma water, the retrogressed uterus over a period of 2 h *in vitro* maintains a substantial concentration gradient, relative to the medium, for both Mg^{2+} (3.8:1 in 1.2 mM, 38:1 in 0.12 mM Mg^{2+}) and K^+ (13:1). Under the conditions of incubation used, the Na^+ content of the uterus was maintained at the preincubation level and was not significantly affected by insulin.

With respect to insulin action, reducing the Ca^{2+} to 1/2 (from 2.54 to 1.27 mM) or the Mg^{2+} to 1/10 (from 1.2 to 0.12 mM) does not affect the capacity of insulin to increase the net accumulation of both Mg^{2+} and K^+.

Extracellular compartment of rat uterus: lack of effect of insulin (Table II)

TABLE II

LACK OF EFFECT OF INSULIN, BUT EFFECT OF OUABAIN, ON THE EXTRACELLULAR SPACE OF RETROGRESSED RAT UTERUS

The sodium space is calculated from the Na^+ content measured in individual hemiuteri following dissolution in HNO_3 (see Table I). The sorbitol space is calculated as the ratio of cpm in the tissue extract to cpm ([^{3}H]sorbitol, 5 μCi per 2.0 ml) in the incubation medium. After 120 min of incubation, uteri were processed and weighed. Explants were then placed in individual grinding tubes and quickly frozen by plunging the tubes into a cold ethanol bath to which dry ice had been added; each tube contained 150 μl of 0.5 M perchloric acid. Subsequent homogenization and related operations were carried out in an ice bath or a cold room maintained at 4 °C. Following homogenization, pestles were rinsed with 200 μl of 0.5 M perchloric acid, samples were centrifuged and a 250-μl aliquot of supernatant from each was then transferred to individual centrifuge tubes. 200 μl of 0.5 M potassium carbonate were added to neutralize the protein filtrate, the contents mixed, and the precipitate ($KClO_4$) centrifuged out. 200 μl of the neutralized supernatant were then added to Bray's solution[7], modified, that contained 1 g of dimethyl-POPOP per l in place of 0.2 g POPOP. To determine the radioactivity in the incubation medium, 150 μl of medium *plus* 850 μl of water were mixed with 5 ml of 0.5 M perchloric acid in a centrifuge tube and the solution then neutralized with 4 ml of 0.5 M potassium carbonate; following centrifugation a 200-μl aliquot of the supernatant was counted as above. Number of hemiuteri analyzed is given in parentheses; an insulin effect on accumulation of K^+ and Mg^{2+} was in each case demonstrated in comparable experiments run concurrently. Numbers of samples and calculations as in Table I.

Ions in medium (mM/l)		*Added to medium*	*Extracellular space (%)*	
Ca^{2+}	Mg^{2+}		*Sorbitol*	Na^+
1.27	0.12	—	53 ± 1.5 (3)	53 ± 2 (6)
		Insulin, 0.1 unit/ml	52 ± 0.5 (3)	50 ± 2 (6)
2.54	0.12	—	52 ± 1.5 (4)	
		Ouabain, $5 \cdot 10^{-4}$ M	47 ± 0.0 (4)	

The increases in Mg^{2+} and K^+ content (insulin effect) of retrogressed uteri incubated *in vitro* under conditions used throughout this investigation are not associated with significant alterations in the sorbitol space (Table II), the Na^+ space (Table III), or the total water of the tissue (Table III). It is our conclusion, therefore, that the increases in Mg^{2+} and K^+ content obtained with insulin in these experiments reflect cation translocation and cannot be ascribed to an alteration in extracellular space.

Perhaps it should be pointed out that the effect of insulin on extracellular space

TABLE III

INSULIN STIMULATION OF Mg^{2+} AND K^+ ACCUMULATION AS INFLUENCED BY OUABAIN AND BY K^+ IN THE MEDIUM

The Ca^{2+} concentration of the medium was 2.54 mM, the Mg^{2+} concentration 0.12 mM, and the substrate 10 mM sodium pyruvate. Hemiuteri were analyzed after dissolution in HNO_3; procedure, number of samples and calculations as in Table I.

Added to incubation medium	*Medium**		*Weight (mg)*		*Ion concentrations (μmoles/g wet wt)*				
	Ouabain	*K^+*	*Wet*	*Dry*	*Found*			*Change*	
					Na^+	*K^+*	*Mg^{2+}*	*Na^+*	*K^+*
No insulin	−	+ (4)	17.6	3.6	61.7 ± 3.6	75.6 ± 3.8	3.87 ± 0.12		
	+	+ (5)	13.4	2.9	100.7 ± 3.0	53.0 ± 1.2	4.73 ± 0.01	+39.0	−22.6
Insulin (0.1 unit/ml)	−	+ (4)	16.3	3.5	62.0 ± 2.0	85.4 ± 2.8	4.57 ± 0.17		
	+	+ (5)	13.0	2.9	100.9 ± 4.3	46.8 ± 1.6	4.90 ± 0.12	+38.9	−38.6
No insulin	−	+ (6)	17.1	3.6	81.7 ± 2.9	80.2 ± 2.1	4.77 ± 0.12		
	−	− (6)	18.2	4.0	116.8 ± 2.1	45.6 ± 2.7	5.23 ± 0.04	+35.1	−34.6
Insulin (0.1 unit/ml)	−	+ (5)	16.1	3.4	80.1 ± 4.6	85.9 ± 1.7	5.43 ± 0.12		
	−	− (6)	18.8	4.0	122.6 ± 2.4	47.1 ± 1.7	5.47 + 0.08	+42.5	−38.8

* Ouabain ($5 \cdot 10^{-5}$ M) added during last 80 min of incubation; K^+ concentration 5.85 mM when present.

appears to be influenced by the peculiarities of the tissue analyzed, by the ion concentration of the incubation medium, and by the choice of substrate. Zierler *et al.*[5] have shown that insulin causes a shift of water from extracellular to intracellular space of skeletal muscle, but only when glucose is present in the medium, not in the absence of added glucose. Crofford and Renold[6] observed no effect of insulin on extracellular space of epididymal adipose tissue. It is emphasized that the increase in K^+ and Mg^{2+} content obtained with insulin in the present experiments was demonstrated with no glucose in the medium.

The average value of 52% reported here for extracellular space in retrogressed uterus agrees well with that obtained by Halkerston *et al.*[8] for the inulin space in this tissue; the ^{22}Na space (78%) obtained by these investigators was substantially greater, and our values for the sodium space throughout the present study varied from 40 to 58%. In addition, the change in the sorbitol space with ouabain was small when compared to the changes in intracellular Na^+ and K^+ (Table III).

(Na^+ + K^+)-ATPase: insulin stimulation of Mg^{2+} and K^+ accumulation as influenced by ouabain and by K^+ in the medium (Tables III and IV)

The function of (Na^+ + K^+)-ATPase with respect to ion transport is inhibited by ouabain and is dependent on the concentration of K^+ in the extracellular fluid[9].

Addition of ouabain to the medium (Table III) gives rise to a number of very striking changes; the Na^+ content of the tissue rises (61.7 up to 100.7 μmoles/g) and the K^+ content decreases (75.6 down to 53.0 μmoles/g). At the same time, the Mg^{2+} content increases to levels as high as that obtained with insulin in the absence of ouabain, and no additional effect can be demonstrated when insulin is added.

Omission of K^+ from the incubation medium (Table III) limits the enhancement by insulin of Mg^{2+} and K^+ accumulation in the isolated uterus. The fact that the final concentration of Mg^{2+} attained with insulin in K^+-free and K^+-containing media is the same suggests that the intracellular capacity for Mg^{2+} does have an upper limit.

TABLE IV

EFFECT OF OUABAIN AND OF INSULIN, ON K^+, Mg^{2+} AND Ca^{2+} CONTENT OF RAT UTERUS

The Ca^{2+} concentration of the medium was 2.54 mM, the Mg^{2+} concentration 0.12 mM, the substrate 10 mM sodium pyruvate. Tissues were analyzed after incineration (600 °C). 3 or 4 hemiuteri were pooled in platinum crucibles and incinerated overnight. After cooling, the ash was dissolved in 100 μl of concentrated HCl, and transferred quantitatively with water rinses to Na^+-free Pyrex tubes (total volume 1.6 ml); 10-μl, 100-μl and 500-μl aliquots were then taken for K^+–Na^+, Mg^{2+} and Ca^{2+} determinations, respectively. Number of hemiuteri used is given in parentheses and calculations are as in Table I. U, control.

Medium for incubation	*Insulin (0.1 unit/ml)*	*Wet weight (mg)*	*Ion concentrations found (μmoles/g wet wt)*		
			K^+	*Mg^{2+}*	*Ca^{2+}*
Control, no ouabain added	− (10)	17.1 ± 0.6	75.4	4.73	3.19
	+ (10)	16.5 ± 0.6	81.6	5.47	3.22
Test, ouabain ($5 \cdot 10^{-4}$ M) added at zero time	− (3)	12.2	52.2	5.35	3.44
	+ (3)	12.4	48.8	5.14	3.37
	U (10)	20.9 ± 1.4	67.9	4.73	2.02

future investigation is whether or not an increase in the concentration of Mg^{2+}, K^+ at specific intracellular loci can activate select enzyme units and, in turn, generate sustained energy trapping and protein synthesis. Regulatory functions for K^+ and Mg^{2+} in non-mammalian cells have been demonstrated. In *Escherichia coli* K^+ is important for growth, and Mg^{2+}-GTPase is coupled with the assembly of ribosomal subunits for protein synthesis[20]. Indeed, the rate of growth of wild-type *E. coli* can be shown to be dependent upon the concentration of Mg^{2+} in the culture medium[21].

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