Insulin stimulation of glucose uptake and the transmembrane potential of muscle cells in culture

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Received 2 July 1986

The membrane potential of L6 muscle cells was measured with the fluorescent dye bis-oxonol. Hyperpolarizations of up to 15 mV were caused by gramicidin (in *N*-methyl-D-glucamine$^+$ medium), or by monensin or ionomycin. Depolarization was achieved with gramicidin (in Na^+ medium), or with K^+. Insulin did not change the resting membrane potential of – 70 mV, yet it effectively stimulated 2-deoxy-D-glucose uptake. Conditions that hyperpolarize the cells did not alter the basal rate of hexose uptake. Moreover, insulin was still capable of stimulating hexose uptake in depolarized cells. It is concluded that modulation of the membrane potential is probably not a signalling event in insulin stimulaton of hexose uptake.

Hexose uptake *Membrane potential* *(1-6 cell)* *Insulin* *Second messenger*

1. INTRODUCTION

Uptake of glucose into muscle is regulated by hormonal factors, predominantly insulin, as well as by the contractile activity of the tissue. The molecular mechanisms that underlie such regulation remain unknown. The mechanism of action of insulin has been more extensively studied in adipose cells. A current hypothesis suggests that in these cells, insulin induces the incorporation of glucose carriers from intracellular membranes into the plasma membrane [1,2]. In addition, it has also been proposed that insulin increases the intrinsic activity of individual carriers at the plasma membrane [3,4]. Translocation of glucose carriers to the surface membrane has also been suggested to take place in the insulin-treated diaphragm [5].

Whether insulin increases the number of carriers or their intrinsic activity, specific signals must transfer information from the hormone-receptor complex to the glucose carriers. The nature of these signals remains unknown. Zierler and Rogus [6] reported an insulin-dependent hyperpolarization of skeletal muscle of about 6–9 mV, but the universality of this response has been contested [7]. Zierler and Rogus [8] have further suggested that the insulin-mediated hyperpolarization may play a signalling role in stimulation of hexose uptake. These authors measured a small but consistent elevation in 2-deoxy-D-glucose uptake in segments of isolated muscles hyperpolarized by immersion in a non-conductive solution and application of a transcellular current. However, a direct cause-effect relationship between the insulin-induced hyperpolarization and the stimulation of hexose uptake has not been demonstrated.

We have previously established that the L6 line of differentiating skeletal muscle cells is a suitable model system for studies on insulin action [9]. L6 cells differentiate in culture from myoblasts into myotubes, which display many of the characteristics of adult skeletal muscle [10]. Furthermore, the cells are amenable to suspension for the determination of intracellular ionic concentration by fluorimetric procedures [11,12]. We have recently used the fluorescent dye bis-oxonol to measure the membrane potential in L6 cells [13]. In the present

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

study we analyze the effect of insulin and of exogenous ionophores on the membrane potential. The effect of modulations of the membrane potential on glucose uptake and its stimulation by insulin are also described.

2. EXPERIMENTAL

2.1. *Materials*

Bis-(1,3-diethylthiobarbituric)trimethine oxonol (bis-oxonol) was purchased from Molecular Probes; ionomycin from Calbiochem; Gramicidin, monensin, porcine insulin, 2-deoxy-D-glucose and cytochalasin B from Sigma. Culture media and sera were from Gibco and 2-deoxy[^{3}H]glucose was from ICN.

2.2. *Solutions*

All solutions contained, in mM: 20 Hepes, 10 D-glucose, 1 $CaCl_2$, 3 KCl and 140 of either sodium chloride (Na-solution), potassium gluconate (K-solution) or *N*-methyl-D-glucamine chloride (NMG-solution). Omission of any of these components is specifically indicated. Gluconate was chosen as the counterion for the K-solution in order to prevent cell swelling, likely to occur in KCl solutions [13]. All solutions were adjusted to pH 7.3 and were made 300 ± 10 mosM. Osmolarity was measured by depression of the freezing point.

2.3. *Cell cultures*

L6 cells were grown in α-minimal essential medium (MEM) in the presence of 2% fetal calf serum as described in [9]. Cultures were studied after onset of cell fusion, when the cells become insulin responsive [9]. For hexose transport measurements, monolayers of L6 cells were incubated in serum-free medium for 5 h in MEM containing 28 mM D-glucose, followed by incubation with or without insulin. Hexose uptake was then determined by incubation with 10 μM [^{3}H]deoxyglucose for 10 min. The facilitated diffusion component of the total uptake was calculated in parallel samples containing 5 μM cytochalasin B. We have shown earlier that initial rates of uptake are measured under these conditions [9,14].

The membrane potential was measured in suspended cells with the fluorescent dye bis-oxonol as described [13]. Approx. 2×10^5 suspended cells were exposed to 200 nM bis-oxonol in the specified solution. Fluorescence was determined at 37°C in a 650-40 Perkin Elmer spectrofluorometer adapted with a magnetic stirrer. Excitation and emission wavelengths were 540 and 580 nm (2 and 5 nm bandpass), respectively. Calibration of the fluorescence as mV of membrane potential was performed by treating the cells with 40 nM gramicidin in solutions of variable Na^+ concentrations (iso-osmotically replaced by *N*-methylglucamine$^+$) (see [13]).

3. RESULTS AND DISCUSSION

3.1. *Modulations of the membrane potential*

The resting membrane potential in L6 cells suspended in Na-solution averaged 68 ± 6 mV (negative inside). Fig.1a shows that addition of monensin in Na-solution caused a rapid hyperpolarization. In the experiment illustrated, the membrane potential increased from -70 mV to -85 mV. Monensin, an electroneutral, monovalent cation-ionophore is expected to exchange extracellular Na^+ for intracellular K^+, resulting in an increased cytoplasmic [Na^+]. Whereas the ionophore action is electroneutral, an increase in

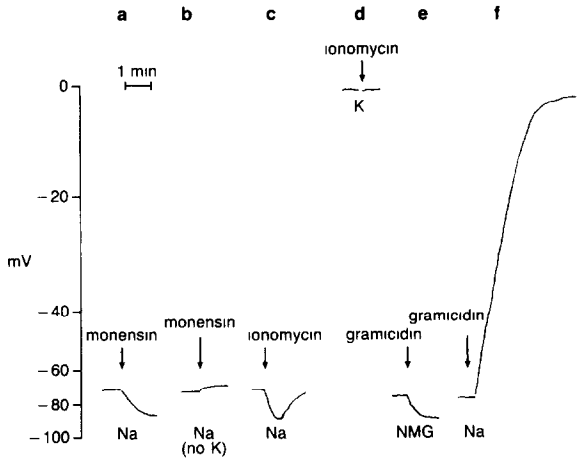

Fig.1. Effect of several ionophores on the membrane potential of L6 cells, measured with the fluorescent indicator bis-oxonol. L6 cells (aprox. 2×10^5) were suspended in either Na-solution, Na-solution devoid of KCl, K-solution or NMG-solution, as indicated, at 37°C. Additions: 2 μM monensin; 250 nM ionomycin; 40 nM gramicidin. The fluorescence was calibrated as in section 2. Results are of one experiment representative of three.

cytoplasmic [Na^+] is expected to stimulate the electrogenic Na^+,K^+-ATPase. This stimulation would result in a hyperpolarization. Consistent with this interpretation, fig.1b shows that the hyperpolarization caused by monensin disappeared in the absence of extracellular K^+.

Fig.1c shows that addition of ionomycin, a Ca^{2+} ionophore, caused a rapid and transient (The repolarization that follows the ionomycin-mediated hyperpolarization could be due to closure of the Ca^{2+}-induced K^+-channels, or to the subsequent opening of Ca^{2+}-induced Cl^- channels, as described for other cells.) hyperpolarization (to −85 mV), consistent with the opening of a Ca^{2+}-induced K^+ conductance [15]. Indeed, the ionomycin-mediated hyperpolarization was not observed in cells suspended in K-solution (fig.1d). In this solution the cells become depolarized (see also [13]) and the K^+ transference number is increased, so that the ionophore has little effect on the membrane potential.

Fig.1e shows that addition of gramicidin to cells suspended in NMG-solution also caused a rapid hyperpolarization (to −83 mV), likely due to the uncompensated outward diffusion of K^+ (and to a lesser extent Na^+) through the channel-former.

In experiments similar to those illustrated in fig.1, decreasing the amount of ionophores resulted in smaller hyperpolarizations. Changes in the membrane potential of $\leqslant 4$ mV were clearly detectable. These results indicate that bis-oxonol can detect small hyperpolarizations in L6 cells.

The last trace in fig.1 shows virtually complete depolarization achieved with gramicidin in Na-solution, consistent with the poor monovalent-cation selectivity of the channel-former.

3.2. *Effect of insulin on the membrane potential*

Fig.2 shows a typical tracing of the membrane potential in L6 cells, before and after addition of insulin (0.1 μM). This concentration of insulin causes maximal stimulation of hexose transport in these cells [9], and stimulation of transport is observed both in cell monolayers and in suspended cells [11]. However, the hormone did not alter detectably the membrane potential within 10 min of addition (fig.2), or even after longer incubations (not shown). In this time period, stimulation of glucose transport is already apparent [9]. Depletion of serum for 5 h prior to addition of insulin

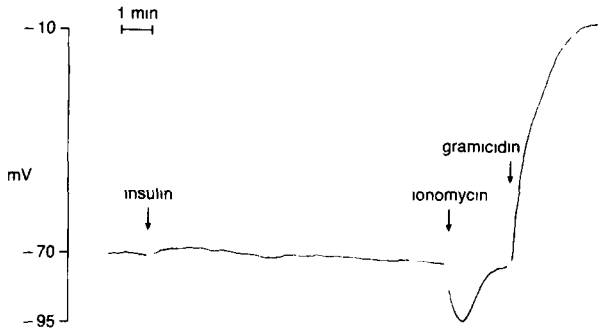

Fig.2. Effect of insulin on the membrane potential of L6 cells. Cells were suspended in Na-solution containing bis-oxonol at 37°C, and the fluorescence was recorded and calibrated as described in section 2. Additions: 0.1 μM insulin; 250 nM ionomycin; 40 nM gramicidin. Results are of one experiment representative of twelve.

gave identical results to those in fig.2. The figure also shows that treatment with insulin dit not prevent the response of the membrane potential to either ionomycin (hyperpolarization) or gramicidin (depolarization), suggesting that hormone does not interfere with the potential-sensitivity of bis-oxonol.

3.3. *Effects of hyperpolarization and of insulin on hexose uptake*

Table 1 shows the effect of monensin, ionomycin and gramicidin (the latter in NMG-solution) on hexose uptake. As described above, these three ionophores hyperpolarize the cells under the conditions used. However, they did not cause a significant stimulation of hexose transport. Moreover, they did not interfere with the subsequent stimulation of hexose transport by insulin. The table also shows the effect of insulin in depolarized cells (in K-solution, or in Na-solution containing gramicidin). The hormone was still capable of eliciting a significant stimulation of hexose transport in these conditions.

These results indicate that insulin per se does not change the membrane potential in L6 cells, that hyperpolarization alone is not sufficient to stimulate hexose uptake, and that the effect of insulin is not prevented by cell depolarization. Therefore, it is unlikely that the stimulation of hexose uptake by the hormone is mediated by modulations of the membrane potential. It is concluded that other signals must mediate between insulin receptors and glucose carriers.

Table 1

Effect of hyperpolarizing and depolarizing conditions on hexose uptake

Condition	2-Deoxyglucose uptake (pmol/min per mg protein)	
	− insulin	+ insulin
Control	12.3 ± 0.5	17.6 ± 1.3
Monensin (10 min)	13.6 ± 0.4	
Ionomycin (10 min)	11.0 ± 0.3	
Ionomycin (30 min)	9.9 ± 1.3	15.6 ± 1.1
NMG-solution	12.5 ± 1.0	17.2 ± 1.3
Gramicidin in NMG-solution (30 min)	13.7 ± 0.5	16.2 ± 0.2
K-solution	15.4 ± 0.5	20.0 ± 1.6
Gramicidin in Na-solution (30 min)	10.7 ± 0.5	13.7 ± 0.7

L6 cells were serum-deprived for 5 h in MEM containing 28 mM D-glucose. They were then incubated for 30 min in the presence or absence of 0.2 μM insulin, 2 μM monensin, 250 nM ionomycin, or 40 nM gramicidin, in culture medium (unless where specified otherwise) The incubation was followed by 2-deoxyglucose uptake determinations for 10 min as in section 2. The solutions of the uptake assay were the same as those of the incubations, but were devoid of D-glucose and contained 10 μM [^{3}H]deoxyglucose. When the ionophores were tested for 10 min, they were present only in the uptake assay. Results are the mean ± SD of at least quadruplicate determinations.

We have previously shown that insulin does not change the cytosolic [Ca^{2+}] in quin 2-loaded L6 cells, and that chelating intracellular Ca^{2+} does not prevent insulin-stimulation of hexose transport [11]. The present study provides three arguments that further rule out a role for cytosolic Ca^{2+} in mediating insulin-stimulation of glucose transport: (i) The Ca^{2+} ionophore ionomycin caused hyperpolarization of L6 cells, and this effect was largely dependent on the presence of extracellular Ca^{2+} (not shown), suggesting that increased cytosolic Ca^{2+} mediated the hyperpolarization. In contrast, insulin did not change the membrane potential, consistent with a lack of effect of the hormone on cytosolic [Ca^{2+}]. (ii) Stimulation of hexose uptake appears to be independent of changes in cytosolic [Ca^{2+}] since ionomycin failed to stimulate deoxyglucose transport. (iii) No stimulation of hexose uptake was observed in depolarizing K-solution. Under these conditions, there is a large increase in cytosolic [Ca^{2+}] which is nifedipine-sensitive and is likely due to opening of voltage-sensitive Ca^{2+} channels [13].

In summary, changes of membrane potential in L6 muscle cells were monitored with the fluorescent indicator bis-oxonol. Insulin did not alter the resting membrane potential within 10 min. Hyperpolarizing conditions did not stimulate hexose uptake in these cells, and depolarizing conditions did not prevent insulin stimulation of hexose uptake. It is suggested that insulin stimulation of sugar transport in muscle cells in culture is not signalled by changes in the membrane potential.

ACKNOWLEDGEMENTS

We thank Dr S. Grinstein for valuable discussion, and E. Mack for participation in the initial stages of this study. This work was supported by the Medical Research Council of Canada. A.K. is the recipient of an MRC Scientist Award.

REFERENCES

[1] Cushman, S.W. and Wardzala, L.J. (1980) J. Biol. Chem. 255, 4758-4762.
[2] Suzuki, K. and Kono, T. (1980) Proc. Natl. Acad. Sci. USA 77, 2542-2545.
[3] Simpson, I.A., Yver, D.R., Hissin, P.J., Wardzala, L.J., Karnieli, E., Salans, L. and Cushman, S.W. (1983) Biochim. Biophys. Acta 763, 393-407.
[4] Hyslop, P.A., Kuhn, C.E. and Sauerheber, R.D. (1985) Biochem. J. 232, 245-254.
[5] Wardzala, L.J. and Jeanrenaud, B. (1981) J. Biol. Chem. 256, 7090-7093.
[6] Zierler, K. and Rogus, E.M. (1981) Biochim. Biophys. Acta 640, 687-692.
[7] Stark, J. and O'Doherty, J. (1982) Am. J. Physiol. 242, E193-E200.
[8] Zierler, K. and Rogus, E.M. (1980) Am. J. Physiol. 239, E21-E29.
[9] Klip, A., Li, G. and Logan, W.J. (1984) Am. J. Physiol. 247, E291-E296.
[10] Shainberg, A., Yagil, G. and Yaffe, D. (1971) Dev. Biol. 25, 1-29.
[11] Klip, A., Li, G. and Logan, W.J. (1984) Am. J. Physiol. 247, E297-E304.
[12] Klip, A., Ramlal, T. and Cragoe, E.J. jr (1986) Am. J. Physiol. 250, C720-C728.
[13] Klip, A., Mack, E., Ramlal, T. and Walker, D. (1986) Arch. Biochem. Biophys. 246, 865-871.
[14] Klip, A., Logan, W.J. and Li, G. (1982) Biochim. Biophys. Acta 687, 265-280.
[15] Lew, V.L. and Ferreira, H.C. (1978) Curr. Top. Membrane Transp. 10, 217-277.