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The dependence on intracellular ATP concentration of ATP-sensitive K-channels and of Na,K-ATPase in intact HIT-T15β-cells

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We have studied the effects of changes of intracellular ATP concentration ($[ATP]_i$) on the activity of ATP-sensitive K-channels ($I_{K(ATP)}$) and of Na,K-ATPase in intact cells of the insulin-secreting cell-line HIT-T15. Pre-exposure of HIT β-cells to oligomycin caused a dose-dependent reduction in $[ATP]_i$. Marked activation of $I_{K(ATP)}$ activity was found when ATP was lowered below 3 mM. Na,K-ATPase was progressively inhibited as ATP was lowered to 1.5 mM. These data demonstrate that changes in intracellular ATP in the millimolar range markedly influence the activity of two β-cell membrane proteins having affinities for ATP in the micromolar range. This suggests that submembrane [ATP] may be considerably below the measured bulk cytosolic concentration. The findings also support the proposed role of intracellular ATP in mediating effects of changes in glucose concentration on the activity of β-cell $I_{K(ATP)}$ and insulin secretion.

ATP-sensitive-K-channel; Potassium channel; Na,K-ATPase; Insulin secretion; (HIT-T15 β-cells, Pancreatic β-cells)

1. INTRODUCTION

Recent studies have identified a key role for ATP-sensitive K-channels ($I_{K(ATP)}$) in control of insulin release from the pancreatic β-cell [1–8]. It is currently envisaged that an increase in extracellular glucose concentration elicits an increased rate of glucose metabolism within the β-cell and that the subsequent increase in intracellular ATP/ADP ratio inhibits $I_{K(ATP)}$ activity. The resulting membrane depolarization opens voltage-dependent Ca-channels and leads to increases in Ca^{2+} influx, intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and insulin secretion.

Despite the considerable evidence that $[ATP]_i$ acts as the primary physiological regulator of $I_{K(ATP)}$ activity [1], there remains a disparity between measured $[ATP]_i$ in the pancreatic β-cell and the very much lower concentration of ATP that maximally inhibits the channel activity in excised membrane patches [9,10]. A number of explanations have been suggested for this discrepancy, in particular the ability of ADP to oppose the effect of ATP on $I_{K(ATP)}$ [11,12] and the fact that even in the unstimulated β-cell the majority of $I_{K(ATP)}$ channels are closed [13]. In the present study we demonstrate that changes in β-cell ATP content in the millimolar range can indeed cause changes in $I_{K(ATP)}$ activity in intact β-cells. We additionally show that another plasma membrane protein, Na,K-ATPase, is also markedly affected in intact β-cells by similar changes in $[ATP]_i$ despite the fact that much lower concentrations of ATP saturate the enzyme in isolated membrane preparations [14]. These data can be explained by postulating that in the β-cell the sub-membrane concentration of ATP is lower than the bulk cytosolic concentration.

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Abbreviations: $I_{K(ATP)}$, ATP-sensitive K-channel; $[ATP]_i$, intracellular ATP concentration; $[Ca]_i$, intracellular free Ca^{2+} concentration; DMSO, dimethylsulfoxide

2. MATERIALS AND METHODS

2.1. *Cell culture*

HIT-T15 β-cells (passage numbers 75–90) were cultured in RPMI 1640 tissue culture medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), fungizone (0.25 μg/ml) and fetal calf serum (10%) at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%) as previously described in detail [15]. Cells were passaged weekly and harvested using trypsin-EDTA. HIT cells were seeded in Multiwell plates at a density of 5×10^5 cells per well (for ^{86}Rb-efflux and ^{86}Rb-uptake studies) and in culture flasks at 3×10^7 cells per flask (for Quin 2 studies). The cells were cultured for 3–6 days before the experiments.

All experiments were performed at 37°C.

2.2. *^{86}Rb-efflux measurements for assay of $I_{K(ATP)}$*

$I_{K(ATP)}$ activity was assayed by measuring glibenclamide-sensitive ^{86}Rb-efflux [16,17]. The day before an experiment, ^{86}RbCl (from Amersham, spec. act. 86–682 Ci/mol) was added to wells (0.1 μCi/well) and cells were loaded with the isotope overnight. On the day of the experiment, the culture medium was removed and replaced with solution A containing (mM): NaCl 124, $CaCl_2$ 1.8, $MgCl_2$ 0.8, KCl 10, Hepes 20 (pH 7.5 with NaOH) to which was added ^{86}RbCl (0.1 μCi/ml). For Ca-free solution (B), Ca was replaced by Mg and 1 mM EGTA was added. To deplete the cells of ATP, prein-

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cubation was carried out for 20 min at 37°C in 1 ml of solution A or B containing ^{86}RbCl (0.1 μCi/ml) in the absence or presence of 0.12–2.4 μg/ml oligomycin and 1 mM 2-deoxy-D-glucose. After the preincubation, the medium was replaced with solution A or B without ^{86}RbCl and the cells were incubated for 10 min in the absence or presence of 1 μM glibenclamide and/or 1 mM ouabain. To determine the cellular content of ^{86}Rb, cells were washed twice with solution A supplemented with 1 μM glibenclamide (to prevent loss of ^{86}Rb via $I_{K(ATP)}$) and then extracted with 1 ml of 0.1 M boric acid (pH 8 with NaOH) and 0.5 M NaCl. The extract was counted using Cerenkov counting. Stock solutions of oligomycin were prepared in dimethylsulphoxide (DMSO) at 2.4 mg/ml and of glibenclamide (Hoechst) at 1 mM in DMSO.

2.3. *^{86}Rb-uptake measurements for assay of Na,K-ATPase*

Na,K-ATPase activity was measured as ouabain-sensitive ^{86}Rb-uptake. HIT cells were preincubated for 20 min in buffer containing (mM): NaCl 134, $CaCl_2$ 1.8, $MgCl_2$ 0.8, Hepes 20 (pH 7.5 with NaOH) in the presence or absence of the ATP-depleting agents described above. K^+ was omitted from the preincubation medium in order to increase the intracellular Na^+ concentration. After preincubation, the cells were further incubated for 5 min in 1 ml of solution C containing (mM): NaCl 129, $CaCl_2$ 1.8, $MgCl_2$ 0.8, KCl 5; Hepes 20 (pH 7.5 with NaOH) with 0.5 μCi ^{86}RbCl in the presence or absence of 1 mM ouabain or 1 μM glibenclamide. After washing twice with 1 ml of solution C, supplemented with 1 μM glibenclamide to prevent Rb loss, ^{86}Rb was extracted and counted as described above.

2.4. *ATP measurements*

To measure $[ATP]_i$, cells incubated under parallel conditions to those used for the ^{86}Rb-flux measurements were extracted in 0.5 ml of 4% perchloric acid. ATP in the extracts was quantitated using a luciferase assay and a commercial luminometer (LKB 1250). The intracellular concentration of ATP was calculated assuming a volume of 1 pl per cell [18].

2.5. *Measurement of intracellular Ca^{2+}*

HIT cells (approximately 5×10^7) were detached with trypsin-EDTA, washed in 20 ml of RPMI medium and resuspended in 4 ml of solution A. Cells were loaded with Quin 2 as previously descdribed [19] and incubated for 20 min. After loading, cells were diluted 4-fold in the medium and incubated for a further 20 min. They were then collected by centrifugation ($190 \times g$ for 5 min), resuspended in a Ca-free solution (B) or Ca^{2+}-containing solution (A) at a density of 25×10^6 cells/ml and stored on ice before use.

Quin 2-loaded HIT cells (5×10^6) were transferred to a cuvette (final vol. 2 ml) in a Perkin-Elmer LS5 luminescence spectrometer and preincubated for 10 min with continuous stirring before addition of ATP-depleting agents. The intracellular free Ca^{2+} concentration was calculated as described in [20].

All data are expressed as mean ± SE for n observations.

3. RESULTS

3.1. *ATP content*

Fig.1 shows the effect of increasing concentrations of oligomycin on $[ATP]_i$ in the presence of 1 mM 2-deoxy-D-glucose. Oligomycin (0.12–2.4 μg/ml) caused a dose-dependent decrease in intracellular concentration of ATP from 5.2 mM in the absence of oligomycin to less than 1 mM at the highest concentration of oligomycin. Neither $[ATP]_i$ nor ^{86}Rb-efflux was influenced by 1 mM 2-deoxyglucose alone (data not shown).

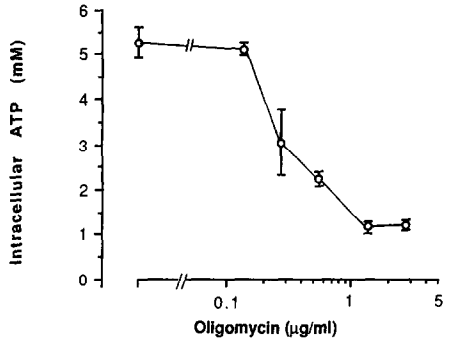

Fig.1. Effect of oligomycin on the intracellular concentration of ATP in HIT cells. ATP was extracted from HIT cells incubated for 20 min in the absence or presence of oligomycin (0.12–2.4 μg/ml) plus 2-deoxyglucose (1 mM) and measured by a luciferase assay. The data are given as mean ± SE for 3–8 observations.

3.2. $I_{K(ATP)}$

A decrease in intracellular ATP concentration increased ^{86}Rb-efflux from HIT cells (fig.2). In non-ATP-depleted cells (data points at >5 mM $[ATP]_i$) ^{86}Rb-efflux amounted to 8% of the initial ^{86}Rb content at the start of the incubation and glibenclamide had little effect. Lowering $[ATP]_i$ below 3 mM progressively increased ^{86}Rb-efflux, up to 6-fold for $[ATP]_i$ less than 1 mM. This augmented ^{86}Rb-efflux was abolished by 1 μM glibenclamide indicating that Rb efflux stimulated by ATP-depletion was through $I_{K(ATP)}$. ^{86}Rb-efflux from ATP-depleted β-cells was not affected by 1 mM ouabain either in the presence or in the absence of glibenclamide (data not shown). Thus under the conditions of these experiments the contribution of Na,K-ATPase to the measured Rb fluxes is negligible.

The effects of ATP-depletion and glibenclamide on ^{86}Rb-efflux during a 10 min incubation were not changed when similar experiments were carried out in the

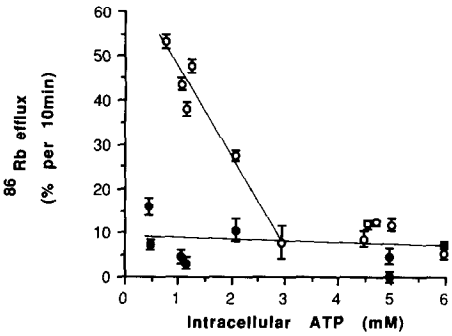

Fig.2. Effect of ATP depletion on ^{86}Rb-efflux from HIT cells. HIT cells were loaded overnight with ^{86}Rb and then pre-incubated for 20 min in the absence or presence of varying concentrations of oligomycin plus 2-deoxyglucose. The ^{86}Rb-efflux from HIT cells during a subsequent 10 min incubation in Rb-free medium with (•) or without (○) 1 μM glibenclamide is expressed as a percentage of the ^{86}Rb content at the start of the incubation. The intracellular ATP concentrations of HIT cells are expressed as an average of the values at the start and the end of the incubation. Each point is the mean ± SE of 4–6 observations.

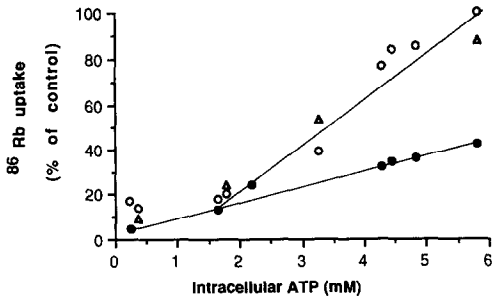

Fig.3. Effect of ATP depletion on ^{86}Rb-uptake by HIT cells. The ^{86}Rb-uptake by HIT cells during a 5 min incubation in Rb-free medium without additions (○) or in the presence of either 1 mM ouabain (●) or 1 μM glibenclamide (△) is expressed as a percentage of the maximal ^{86}Rb-uptake. The intracellular ATP concentration of HIT cells is expressed as an average of the values at the start and the end of the incubation. Each point is the mean of 4 observations (SEs were less than the size of the symbols).

absence of extracellular Ca^{2+}. Thus ^{86}Rb-efflux from non-ATP-depleted cells ($[ATP]_i$ = 5.54 ± 0.43 mM, n = 3) was 14.4 ± 1.5% (n = 5) in Ca-free buffer and 18.3 ± 1.8% (n = 4) in solution A. In cells depleted with 1 mM 2-deoxyglucose plus 1.2 μg/ml oligomycin ($[ATP]_i$ = 0.44 ± 0.08 mM, n = 3) the increase in ^{86}Rb-efflux observed in Ca-free buffer (to 35.1 ± 3.5%, n = 4) was similar to that in solution A (to 34.2 ± 3.1%, n = 4). The increased ^{86}Rb-efflux elicited by ATP depletion in Ca-free buffer was also inhibited by 1 μM glibenclamide (to 16.2 ± 1.9%, n = 4).

3.3. *Na,K-ATPase*

^{86}Rb-uptake via Na,K-ATPase in HIT cells was also modified by changes in $[ATP]_i$ in the millimolar range (fig.3). ^{86}Rb-uptake in the absence of ATP-depletion (data points at 6 mM $[ATP]_i$) was inhibited by 60% by addition of 1 mM ouabain. As $[ATP]_i$ was lowered, ouabain had progressively less effect on ^{86}Rb-uptake with no detectable inhibition for $[ATP]_i$ less than 1.5 mM. The addition of 1 μM glibenclamide to the incubation media did not affect the inhibition of ^{86}Rb-uptake by ATP-depletion indicating lack of significant flux through $I_{K(ATP)}$ under these conditions.

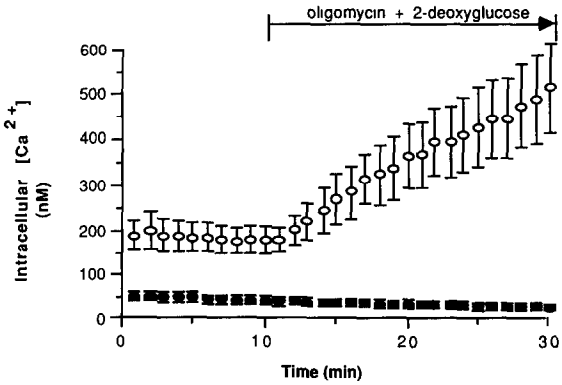

Fig.4. Effect of ATP depletion on the intracellular Ca^{2+} concentration in HIT cells. Intracellular Ca^{2+} concentrations were estimated with Quin 2. After Quin 2/AM loading, the cells were resuspended and incubated in buffer containing 1.8 mM Ca (○) or in Ca-free buffer (●). Oligomycin (1.2 μg/ml) and 2-deoxyglucose (1 mM) were added to the media after 10 min and the incubation was continued for a further 20 min. Each point is the mean ± SE of 3 observations.

3.4. *Intracellular free Ca^{2+}*

Fig.4 shows that $[Ca]_i$ in HIT cells incubated in the presence of extracellular Ca^{2+} was 180 nM; a gradual increase in $[Ca]_i$ was observed on ATP depletion which reached 516 ± 100 nM (n = 3) at the end of 20 min incubation with oligomycin plus 2-deoxyglucose. In Ca-free buffer, however, the basal $[Ca]_i$ (50 nM) was not increased by the addition of the ATP-depleting agents.

4. DISCUSSION

The cloned β-cell line HIT-T15 provides an attractive model for studying the regulatory mechanisms of the pancreatic β-cell because it retains good secretory responses to agents modifying insulin release from normal β-cells including the main physiological regulator glucose and the sulfonylureas used in the treatment of non-insulin-dependent diabetes mellitus [15,18,19]. As in normal β-cells, this cell line possesses an $I_{K(ATP)}$ which is markedly inhibited by micromolar concentrations of ATP in excised membrane patches [21]. Using this β-cell line, we have now established the ATP-dependence for the activities of $I_{K(ATP)}$ and of Na,K-ATPase in intact cells.

We have used glibenclamide-sensitive ^{86}Rb-efflux as a measurement of whole cell $I_{K(ATP)}$ activity [16,17]. We can discount the possibility that significant glibenclamide-sensitive ^{86}Rb-efflux flows through Ca-activated K-channels for two reasons. First, patch-clamp studies have shown that these channels are insensitive to sulfonylureas [4,22]. Secondly, we show here that although $[Ca]_i$ rises on ATP-depletion this effect is only seen in the presence of extracellular Ca^{2+}, whereas omission of external Ca^{2+} did not modify the glibenclamide-sensitive ^{86}Rb-efflux evoked by ATP-depletion. The fact that insulin secretion is inhibited by ATP depletion [23] despite the increase in intracellular Ca^{2+} we report here suggests that a certain minimum concentration of ATP is necessary for the secretory process itself.

The present Rb-efflux study is in agreement with an earlier study on RINm5F cells [17] in showing that the ATP concentration which is required for inhibition of $I_{K(ATP)}$ in intact β-cells is about two orders of magnitude higher than that reported for isolated membrane systems [9,10]. One possible explanation for the discrepancy is that ADP modulates the ATP sensitivity of the channel [11,12]. An alternative possibility is that the sub-membrane ATP concentration may be significantly lower than the value derived from whole cell measurements as previously shown for hepatocytes

[24] and oocytes [25]. Were such an ATP gradient to exist, other membrane proteins would also be exposed to an $[ATP]_i$ lower than the average intracellular concentration. To test this, we examined the effect of ATP-depletion on Na,K-ATPase activity in intact β-cells. This plasma membrane enzyme is known to be activated by micromolar concentrations of ATP when studied in isolated membrane systems [14]. Our data demonstrate that the activity of the Na,K-ATPase in intact β-cells was also sensitive to changes in $[ATP]_i$ within the millimolar range. The β-cell Na,K-ATPase showed a progressive and non-saturating dependence on $[ATP]_i$ within the range 2–6 mM. Below about 2 mM $[ATP]_i$ there was no effect of ouabain on ^{86}Rb-uptake indicating that the β-cell Na,K-ATPase was essentially inactive. Similar findings have been reported for hepatocytes [24].

These data are thus consistent with the concept that the actual concentration of ATP beneath the β-cell plasma membrane is considerably lower than that expressed as an averaged intracellular concentration. Such an intracellular gradient of ATP might arise from unequal rates of ATP supply by mitochondria and utilization by membrane ATPases.

Our findings thus support the view that a major physiological determinant of $I_{K(ATP)}$ activity is the submembrane ATP concentration; however, additional regulatory mechanisms cannot be ruled out.

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