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EARLY STIMULATION OF ATP TURNOVER BY EGF + INSULIN. RELATION TO EXTERNAL pH AND $Na^+|H^+$ EXCHANGE SYSTEM

Saliha TALHA and Louise HAREL

Institut de Recherches Scientifiques sur le Cancer,
B.P. n° 8 - 94802 Villejuif Cedex, France

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We previously shown a rapid increase in ATP turnover after addition of epidermal growth factor and insulin to quiescent 3T3 cell cultures. Here, the relationship between this increase in ATP turnover and the activation by growth factors of $Na^+|H^+$ and $Na^+|K^+$ exchange systems was studied. Our results show that alkalinization of the medium enhances ATP turnover but they do not support the assumption that stimulation by growth factors of the $Na^+|H^+$ exchange induces an increase in ATP turnover since this increase was not inhibited by amiloride. Conversely, when ATP synthesis was abolished, the increase, in intracellular pH, by growth factors, was significantly decreased.

The addition of growth factors to quiescent cell cultures stimulates various early biochemical events which precede DNA replication and cell division (1-4). Among such early events, is the activation of the amiloride-sensitive $Na^+|H^+$ exchange which stimulates ouabain-sensitive $Na^+|K^+$ ATPase (5-11). The possibility that the increase in intracellular pH (pHi), following activation of the $Na^+|H^+$ exchange, represents a compensatory system for H^+ formation by cell metabolism or, on the contrary, that intracellular alkalinization stimulates cell metabolism and in particular, glycolysis, has been discussed (7,10).

We demonstrated (12, 13) an early increase in ATP turnover after addition of epidermal growth factor (EGF) and insulin either alone or in combination to quiescent 3T3 cells. This stimulation may occur either by the glycolytic or the oxidative phosphorylation pathway, and we showed that it was a consequence of an increase in ATP degradation (12). It was thus of interest to verify whether the increase in ATP turnover was a consequence of the stimulation of $Na^+|H^+$ exchange. We studied the effects of extracellular pH and amiloride on the stimulation by growth factors of phosphate uptake,

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phosphate incorporation in small organic acid soluble compounds (Po pool) and ATP labelling . Conversely, we also looked for modifications in $Na^+|H^+$ and $Na^+|K^+$ exchange activities induced by inhibition of ATP turnover.

Materials and Methods

Cells and culture conditions. Swiss 3T3 cells (American Type Culture Collection) were seeded and rendered quiescent as previously described (12).

Phosphate metabolism determination. Quiescent 3T3 cell cultures received EGF (12ng|ml, Collaborative Research) + insulin (2 µg|ml, Calbiochem) 2 min before labelling with $^{32}PO_4H_3$ (^{32}P, 3 µCi|ml) for 15 min. Cells were washed with PBS and treated with 10% perchloric acid (PCA) (12). Inorganic phosphate (Pi) and Po pool of the acid-soluble extract were separated by the butanol method (14) and their radioactivities were determined (12). The protein content of acid-insoluble material was determined by Lowry's method (15) as described before (12). ATP labelling was determined as previously described (12). Results are expressed by nmoles phosphate incorporated|mg protein|15 min, taking into account the specific activity of the incubation medium. Each result represents the average of duplicate determinations.

$^{86}Rb^+$ influx measurements. Quiescent 3T3 cell cultures received 12 ng|ml EGF + 2 µg|ml insulin 1 min before labelling with $^{86}Rb^+$ (4 µCi|ml) for 5 min. After incubation, $^{86}Rb^+$ influx was determinated as described (16). Results are expressed by nmoles $^{86}Rb^+$ incorporated|mg protein|5 min, taking into account the specific activity of the incubation medium. Each result represents the average of duplicate determinations.

Intracellular pH determination. Quiescent 3T3 cell cultures were loaded with the fluorescent pH indicator BCECF and the pHi-dependent emission intensity from the cells was continuously monitored as previously described by Moolenaar et al. (9).

Results

Stimulation by EGF + insulin of ATP synthesis : pH effect. Dense quiescent 3T3 cell cultures were labeled with ^{32}P in the absence or presence of EGF + insulin at different pH between 6.6 and 7.8. Increase in extracellular pH (particularly from 7.4 to 7.8) enhanced phosphate uptake (Fig.1A) and phosphate incorporation in the Po pool (Fig.1B) in non-stimulated cells and decreased the percent stimulation of phosphate uptake and phosphorylation of the Po pool induced by growth-factor addition.

Results of another experiment (table 1), showed that the alkalinization of the incubation medium from 7.4 to 7.8 increased ATP labelling in control cells. The percent stimulation of ATP synthesis by EGF + insulin decreased at pH 7.8 compared to the percent stimulation at pH 7.4.

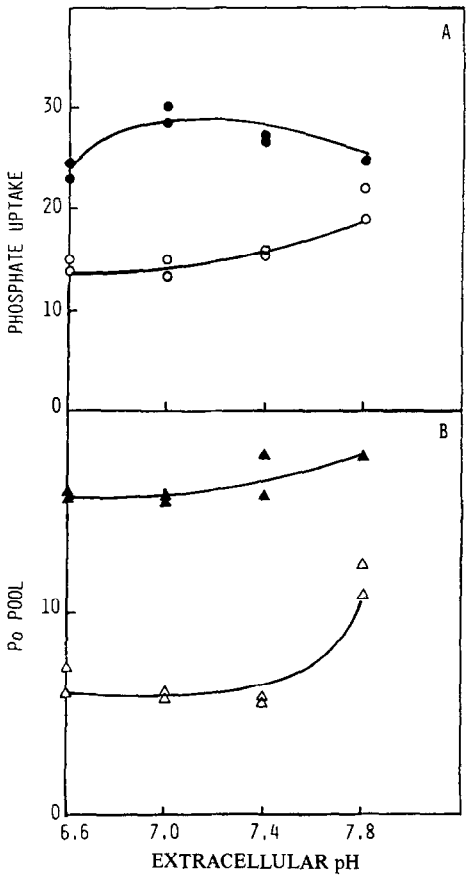

Figure 1 : Influence of extracellular pH on phosphate uptake and Po labelling (typical results). Quiescent 3T3 cell cultures were incubated in fresh medium (adjusted at different pH between 6.6 and 7.8) in the absence or presence of EGF + insulin and labeled with ^{32}P. Phosphate uptake in ●—●, stimulated and o—o, unstimulated cells, Po pool in ▲—▲, stimulated and △—△ unstimulated cells were determined.

Amiloride effect upon stimulation by EGF + insulin of ATP turnover. Dense quiescent 3T3 cell cultures were incubated as in the preceding experiment at pH 6.6 or 7.4 in the presence or absence of amiloride (Merck Sharp et Dohme Chibret) and EGF + insulin. In the presence of amiloride (table 2), and particularly at pH 6.6, phosphate uptake and Po labelling were slightly increased in stimulated cells and, by contrast, slightly decreased in non-stimulated cells. Therefore, the stimulation by EGF + insulin of phosphate uptake and Po labelling was increased in the presence of amiloride at pH 6.6 or 7.4. The effect of amiloride on stimulation by EGF + insulin of ATP turnover was more significant than it was on Po labelling since amiloride more

Table 1 : Influence of extracellular alkalinization upon ATP turnover

Phosphate incorporated in	Extra-cellular pH	- Growth factors	+ Growth factors	Variation (%)
ATP	7.4	2.3 + 0.2	8.4 + 0.1	+ 265
	7.8	3.9 + 0.2	9.8 + 0.1	+ 151
	variation (%)	+ 69	+ 16	
Po pool	7.4	4.6 + 0.1	15.2 + 0.3	+ 230
	7.8	6.2 + 0.3	17.3 + 0.1	+ 179
	variation (%)	+ 34	+ 14	
Acid-soluble fraction	7.4	10.3 + 0.9	23.7 + 0.1	+ 130
	7.8	12.2 + 0.7	24.6 + 1.0	+ 101
	variation (%)	+ 18	+ 4	

Experimental conditions were similar to those described in figure 1, except that medium was adjusted at pH 7.4 and 7.8.
ATP, Po pool and phosphate uptake (acid-soluble fraction) were determined.

sharply decreased ATP than Po labelling in non-stimulated cells (table 2).

From these results, we conclude that stimulation by EGF + insulin of ATP turnover was not a consequence of activation of the $Na^+|H^+$ exchange.

Ouabain effect on the stimulation by EGF + insulin of ATP turnover. In this experiment, dense quiescent 3T3 cell cultures were incubated in the absence or presence of EGF + insulin with or without ouabain (Sigma) and labeled with ^{32}P. The stimulatory effects of EGF + insulin on ATP turnover in the absence and presence of the drug were compared.

Results are shown in table 3. Phosphorylation of Po pool in stimulated and unstimulated cells was not significantly changed by the presence of ouabain. This drug was no longer able to inhibit stimulation by EGF + insulin of ATP turnover.

Table 2 : Influence of amiloride on the increase by EGF + insulin in ATP turnover

Extra-cellular pH	Phosphate incorporated in	Amiloride	- Growth factors	+ Growth factors	Variation (%)
7.4	ATP	-	1.9 + 0.0	6.1 + 0.0	+ 221
		+	1.6 + 0.0	6.1 + 0.3	+ 281
	Po pool	-	5.7 + 0.1	16.8 + 1.0	+ 194
		+	5.2 + 0.2	17.5 + 0.2	+ 236
	acid-soluble fraction	-	15.9 + 0.1	27.4 + 0.1	+ 72
		+	15.1 + 0.1	28.4 + 1.5	+ 91
6.6	ATP	-	3.8 + 0.2	8.0 + 0.2	+ 110
		+	2.1 + 0.1	7.5 + 0.0	+ 257
	Po pool	-	6.7 + 0.6	15.8 + 0.1	+ 136
		+	5.1 + 0.3	16.6 + 0.9	+ 225
	acid-soluble fraction	-	14.6 + 0.6	24.0 + 0.7	+ 64
		+	14.1 + 0.1	28.9 + 0.1	+ 105

In this experiment, fresh medium was adjusted at pH 6.6 and 7.4 and cells were incubated in the absence or presence of EGF + insulin as described in figure 1, except that part of the cultures received amiloride (0.4 mM) simultaneously with growth factors. Phosphate incorporation in ATP, Po pool and acid-soluble fraction was determined.

Table 3 : Influence of ouabain on stimulation by EGF + insulin of ATP turnover

Phosphate incorporated in	Incubation	- Growth factors	+ Growth factors	Variation (%)
ATP	- Ouabain	4.9 + 0.5	13.3 + 0.6	+ 171
	+ Ouabain	3.9 + 0.5	12.9 + 0.4	+ 230
Po pool	- Ouabain	7.9 + 0.1	24.8 + 1.0	+ 214
	+ Ouabain	7.5 + 0.0	22.1 + 0.8	+ 194
Acid-soluble fraction	- Ouabain	15.5 + 1.5	37.5 + 2.0	+ 142
	+ Ouabain	16.5 + 2.0	33.5 + 1.0	+ 103

Quiescent 3T3 cell cultures were incubated in the absence or presence of EGF + insulin with or without ouabain (2mM) and labeled with ^{32}P. Phosphate incorporation in ATP, Po pool and acid-soluble fraction was determined.

Effect of the change in ATP turnover on stimulation by EGF + insulin of $^{86}Rb^+$ uptake and $Na^+|H^+$ exchange. In the experiment shown in table 4, we tried to verify whether inhibition of ATP turnover affected the stimulatory effect of EGF + insulin upon $^{86}Rb^+$ uptake. $^{86}Rb^+$ uptake was determined in the presence or absence of EGF + insulin and oligomycin (Sigma). The effect of oligomycin on ATP labelling was verified simultaneously.

Table 4 : Relationship between ATP synthesis and stimulations by EGF + insulin of $^{86}Rb^+$ influx and $Na^+|H^+$ exchange system

Incubation	ATP LABELLING			$^{86}Rb^+$ UPTAKE		
	- Growth factors	+ Growth factor	Variation (%)	- Growth factors	+ Growth factors	Variation (%)
+Glucose						
-oligomycin	2.6+0.2	8.5+0.1	+ 227	76.2+1.5	113.9+1.2	+ 49
+oligomycin	0.8+0.0	4.5+0.3	+ 462	56.3+2.8	78.7+0.4	+ 39
variation(%)	- 69	- 47		- 26	- 31	
-Glucose						
-oligomycin	2.5+0.1	6.7+0.2	+ 168	81.4+2.9	102.2+3.9	+ 25
+oligomycin	0	0	0	44.4+3.1	50.4+0.2	+ 13
variation(%)	- 100	- 100		- 45	- 50	

Incubation	INTRACELLULAR pH				
	- Growth factors	5 % FCS	$\triangle$ pH	+ EGF + insulin	$\triangle$ pH
Complete medium	7.15	7.32	+ 0.17	7.25	+ 0.10
Glucose-free medium + oligomycin	7.14	7.14	0	7.14	0

Cells maintained for 24 hours in serum-free medium with or without glucose were incubated in the presence or absence of EGF + insulin. In one experiment, cells were labeled either with ^{32}P or with $^{86}Rb^+$ and part of the cultures received oligomycin (5ug|ml) simultaneously with EGF + insulin. ATP labelling and $^{86}Rb^+$ uptake were determined. In another experiment pHi was determined, in the absence or presence of either 5% fetal calf serum (FCS) or EGF + insulin, in quiescent 3T3 cells either in complete medium or in glucose-free medium supplemented with oligomycin.

When oligomycin was added in glucose-free medium, ATP labelling was abolished, as expected, and stimulation by EGF + insulin of $^{86}Rb^+$ uptake was significantly decreased. When oligomycin was added in complete medium, (conditions which allow ATP synthesis by the glycolytic pathway), $^{86}Rb^+$ uptake, like ATP synthesis, was inhibited in control and stimulated cells. However, stimulation, by EGF + insulin, of ATP labelling and $^{86}Rb^+$ uptake remained significant. These results suggest that $^{86}Rb^+$ uptake is regulated by the rate of ATP synthesis.

Results (table 4) concerning the effect of oligomycin on $Na^+|H^+$ exchange show that inhibition of ATP synthesis (by oligomycin in glucose-free medium) also abolished cell alkalinization by EGF + insulin and suggested that the $Na^+|H^+$ exchange also depends on the ATP synthesis. The increase in pHi due to addition of EGF + insulin was unaltered in quiescent cells incubated either in the absence of oligomycin in glucose-free medium or in the presence of oligomycin in complete medium (results not shown).

Discussion

The present results confirm our previous findings (12) that addition of EGF + insulin to quiescent 3T3 cell cultures rapidly increases ATP turnover. They show that in unstimulated cells alkalinization of the medium enhanced ATP turnover, and support the assumption (7,10) that alkalinization of cells enhances cell metabolism. However, our results were unable to demonstrate that the increase by EGF + insulin in ATP turnover was a consequence of an enhancement in the $Na^+|H^+$ exchange activity, since amiloride did not prevent the increase in ATP turnover. By contrast, at pH 6.6, stimulation by EGF + insulin of ATP labelling increased in the presence of amiloride which decreased ATP labelling more in unstimulated than in stimulated cells.

Ouabain did not prevent stimulation by EGF + insulin of ATP labelling. Furthermore, we verified, in our experimental conditions, that amiloride and ouabain were able to inhibit the stimulatory effect of EGF + insulin upon $Na^+|K^+$ ATPase activity (results not shown). Therefore our findings support the

assumption that the stimulation of $Na^+|K^+$ exchange, but not of ATP turnover, is a consequence of the increase by EGF + insulin in the $Na^+|H^+$ exchange activity. Conversely, when ATP synthesis was abolished (by addition of oligomycin in glucose-free medium), then the increase by EGF + insulin in pHi was prevented and stimulation of $^{86}Rb^+$ uptake was significantly decreased. Our results agree with recent data (17) demonstrating that $Na^+|K^+$ pump activity is dependent upon the ATP synthesis in the cells.

It should be noted that addition of platelet-derived growth factor (PDGF) to NR6 cells increased pHi with a lag period of 2 min. On the contrary, in the presence of amiloride, the addition of PDGF produced a rapid cytoplasmic acidification (18). Therefore, these results, like our own, do not support the assumption that stimulation by growth factors of the $Na^+|H^+$ exchange induces an enhancement in ATP turnover and cell metabolism. By contrast, they agree with the hypothesis that the stimulation of $Na^+|H^+$ exchange may be the result of either a change in affinity of the $Na^+|H^+$ exchange for intracellular H^+ (19) or an increase in proton production due to the stimulation of ATP turnover.

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