RECIPROCAL CONTROL OF MEMBRANE PERMEABILITY BY ATP IN 3T6 CELLS: EFFECT OF DIAMIDE

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1. Introduction

Addition of ATP to cultures of mouse transformed cells causes a striking increase in passive membrane permeability. Brief exposure of monolayers of 3T6, SV-40-3T3 or Py-3T3 cells to ATP caused a massive efflux of nucleotide pools labeled with [^{3}H]uridine or [^{3}H]-adenosine, of sugar phosphates and of ions [1,2]. Treatment with ATP allows the entry of normally impermeable molecules like *p*-nitrophenylphosphate, glucose-6-phosphate or NAD [3–5]. These effects were readily reversible and the treated cells grow normally afterwards [1]. The effect of exogenous ATP depends on the concentration of intracellular ATP; a reduction of intracellular ATP leads to a marked increase in the sensitivity of the cells to exogenous ATP [2]. The biochemical basis of this effect may be the phosphorylation of a membrane protein which plays a critical role in the control of the passive permeability of the cell [2].

Diamide (diazene-dicarboxybic acid bis (*N*, *N*-dimethylamide)) [6] is a specific inhibitor of cellular protein kinases from different sources [7–9]. Hence, this compound may provide a powerful tool to explore the role of protein kinase activity in cellular regulation. These considerations prompted us to investigate the effect of diamide on ATP modulation of membrane permeability in cultures of mouse 3T6 cells. We found that diamide exerts a potent synergistic effect with external ATP. The basis of this effect in our system appears to be a marked reduction in cellular ATP levels rather than a selective inhibition of protein kinase activity.

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2. Materials and methods

2.1. *Cells*

Swiss mouse 3T6 cells [10], propagated as in [11] were subcultured into 33 mm Nunc dishes in 0.5% fetal calf serum.

2.2. *Measurement of efflux of acid-soluble pools*

The cells were labeled in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The growth medium was replaced by 1 ml fresh medium containing [^{3}H]nucleoside (0.5 μCi/ml) at 1 μM final conc. After 3–6 h in a humidified atmosphere of 10% CO_2, 90% air at 37°C, the cells were washed 4 times with Dulbecco's modified Eagle's medium and once with medium A, whose composition is as follows: Tris–HCl, 0.1 M; NaCl, 0.05 M; $CaCl_2$, 50 μM; dextran 500, 5.0 mg/ml; pH 8.2 (at 23°C). The cells were incubated at 37.5°C with 1 ml medium A (pH 8.2) in the absence or presence of ATP, diamide or both to cause efflux of acid-soluble pools. The supernatant fluid was removed and radioactivity measured as in [1,2].

2.3. *Preparation of cellular homogenates*

The petri dishes were placed on ice and the cells were washed 3 times with 0.14 M NaCl at 4°C. The cells were then removed with a rubber policeman and pelleted by centrifugation at 750 × *g* for 3 min. Per 10^6 cells, 25 μl of 10 mM Tris–HCl (pH 7.2), 2 mM $MgSO_4$ and 10 mM KCl were added to the cell pellet. After dispersion of the pellet the cells were allowed to 'swell' for 10 min, then disrupted with 100 strokes in a Dounce homogenizer. This material was centrifuged at 20 000 × *g* for 20 min and the enzymes were assayed in the supernatant.

2.4. *Assay of glycolytic enzymes and protein kinase activity*

The glycolytic enzymes were assayed by measuring the disappearance of NADH spectrophotometrically at 340 nm at room temperature. The conditions for the assay of phosphofructokinase [11], aldolase [12], pyruvate kinase [13] and lactate dehydrogenase [11] were as described. Protein kinase activity was measured according to [14]. Protein was determined colorimetrically [15] using crystalline serum albumin as standard. Lactate was measured according to [16]. ATP levels were measured as in [2].

3. Results and discussion

Fig.1 shows that addition of either 0.7 mM diamide or 50 μM ATP to cultures of 3T6 cells incubated at pH 8.2 in medium A (see section 2) produces an increase in efflux of [^{3}H]uridine-labeled pools after a lag of 5 min. In contrast, when ATP and diamide are added together there was a striking synergistic effect which was evident at 5 min, a time at which the individual components were ineffective (fig.1A). The synergistic interaction between diamide and external ATP in increasing the passive permeability of the plasma membrane to [^{3}H]uridine labeled metabolites can be clearly demonstrated at pH 7.3 (fig.1B). At this pH value neither ATP nor diamide elicit any increase in efflux even after 40 min incubation. However, when present together, ATP and diamide markedly increase the exit of [^{3}H]uridine labeled pools. Similar synergistic effects were found when diamide was added at 0.4 or 3 mM instead of 0.7 mM and when the preincubation with diamide was varied from 7–15 min (not shown). These results clearly show that diamide and ATP act synergistically in increasing the passive permeability of the cell membrane to metabolites which normally remain inside the cell.

We have shown that cultures permeabilized by addition of ATP can be brought back to a state of low membrane permeability (sealing) by replacing medium A with a solution of neutral pH containing divalent cations, and a source of energy like glutamine [3] or glucose [2]. Diamide opposes the sealing reaction, as shown in fig.1C.

The foregoing results prompted us to investigate the effect of diamide on the activity of the protein kinase because diamide has been proposed to act as a

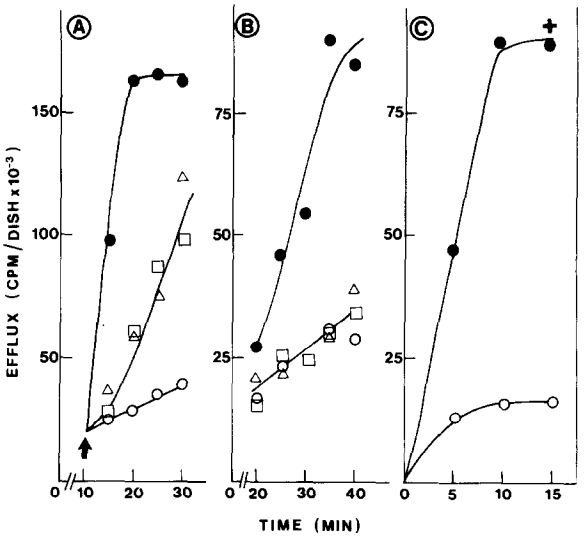

Fig.1. Effect of ATP and diamide on efflux of acid-soluble pools labeled with [^{3}H]uridine (A,B) and resealing (C) of 3T6 cells. Cultures of 3T6 cells with acid-soluble pools labeled with [^{3}H]uridine produced as in section 2 were washed 4 times with NaCl 0.15 M, $CaCl_2$ 50 μM at 37°C, once with medium A (pH 8.2) then incubated with 1 ml medium A (pH 8.2) containing additions as indicated by the following symbols. (A): (○) none; (□) 60 μM ATP; (△) 0.7 mM diamide; (●) 60 μM ATP + 0.7 mM diamide. Diamide was added at the time 0 while ATP was added 10 min after the onset of the incubation as indicated by the arrow. (B): The experimental conditions and symbols are identical to those in (A) except that the medium was pH 7.3 instead of 8.2 and that the preincubation with diamide was 20 min instead of 10 min. (C): Effect of diamide on the resealing of 3T6 cells. Cultures labeled with [^{3}H]uridine were washed and treated with 0.5 mM ATP in medium A (pH 8.2) which causes a massive increase in passive permeability. After 5 min at 37°C, the medium was replaced by the same medium but without ATP (+), or by medium A containing 1 mM $MgCl_2$, 20 mM glucose and 20 mM P_i (pH 8.2) in the absence (○) or in the presence (●) of 0.7 mM diamide. Dishes were removed at intervals and the supernatant fluid was analyzed for radioactivity as in section 2.

selective inhibitor of this enzyme [7–9] and because phosphorylation–dephosphorylation reactions have been suggested to play a role in the permeabilising effect of ATP [2]. We found that exposure of 3T6 cells to 0.7 mM diamide in medium A does cause a decrease (~25%) in protein kinase specific activity as reported in other cell types [8]. However, an identical concentration of diamide causes much more striking decreases in the activity of the phosphofructokinase, pyruvate kinase and aldolase. The inactivation of

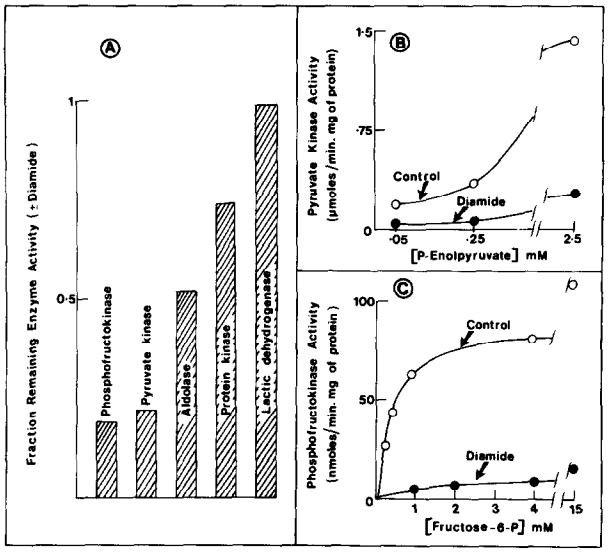

Fig.2. Effect of diamide on the activity of some glycolytic enzymes. (A): Cultures of 3T6 cells were washed with NaCl 0.15 M, CaCl 50 μM 4 times and incubated in medium A (pH 8.2) for 20 min at 37°C in the absence or in the presence of 0.7 mM diamide. At this time the extracts were prepared and specific activities were measured as indicated in section 2. The bars represent the ratio of specific activity with or without diamide. (B): Velocity of pyruvate kinase as a function of phosphoenolpyruvate concentration. (C): Velocity of phosphofructokinase as a function of fructose-6-phosphate concentration in extracts prepared from control and diamide treated cultures of 3T6 cells. The conditions of treatment were as in (A). All other experimental details as in section 2.

pyruvate kinase (fig.2B) and phosphofructokinase (fig.2C) has been verified at different concentrations of phosphoenolpyruvate and fructose-6-phosphate, respectively. Accordingly, diamide reduces the lactate production by intact 3T6 cells (table 1). These results clearly indicate that diamide is a non-specific inhibitor of protein kinase activity. Hence, it is unlikely that the mechanism of action of diamide in our system is solely mediated via inactivation of protein kinase activity.

The increase in membrane permeability produced by external ATP is synergistically potentiated by agents that lower intracellular ATP concentration [2]. Thus an alternative mechanism of action of diamide in increasing the permeabilising effect of ATP could be due to it lowering internal ATP levels. To test this possibility directly, we measured ATP content in 3T6 cells exposed to 0.7 mM diamide. We found that diamide causes a striking decrease (~75%) of internal

Table 1
Diamide decreases lactate production in cultures of 3T6 cells

Treatment		Lactate production ($\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$)
Stage I (10 min)	Stage II (30 min)	
Medium A	Medium A	3.7 ± 0.1
Diamide	Medium A	1.5 ± 0.3
Diamide	Diamide	0.6 ± 0.3
Medium A	Diamide	0.7 ± 0.4

Cultures of 3T6 cells were washed 3 times with 0.15 M NaCl, 50 μM $CaCl_2$ at 37°C. Then the cultures were incubated with 1.5 ml of medium A (pH 8.2) with or without 0.7 mM diamide for 10 min (stage I). After this incubation the cultures were washed twice and incubated in the presence of 20 mM glucose with or without 0.7 mM diamide (stage II). After 30 min incubation the medium was collected for lactate measurements. The results are mean value ± SE of 3 determinations. Similar results were found in separate experiments in which the length of incubation of stage II was 10 min instead of 30 min. Lactate production is linear over 120 min

ATP. This effect was similar to that produced by 2 μg/ml rutamycin, a potent inhibitor of mitochondrial ATPase activity, which was tested for comparison (table 2). This decrease is sufficient to account for the synergistic effect between low concentrations of external ATP (50 μM) and diamide under our experimental conditions. This interpretation is in line with recent findings involving a large series of uncouplers, energy transfer inhibitors or inhibitors of respiration all of which have in common the ability of lowering the cellular content of ATP and of drastically potentiating the stimulation of efflux of phosphorylated metabolites by low concentrations of external ATP [2].

These results show that diamide causes a large

Table 2
Effect of diamide and rutamycin on cellular ATP content in cultures of 3T6 cells

Additions	ATP content (nmol/mg protein)
–	24.4 ± 2.7
Rutamycin, 2 μg/ml	5.1 ± 1.2
Diamide, 0.7 mM	6.6 ± 0.9

Cultures of 3T6 cells were washed 3 times with 0.15 M NaCl, 50 μM $CaCl_2$ at 37°C. Then the cultures were incubated with 1.5 ml medium A for 20 min. At the end of incubation the ATP content of the cultures was measured as in [2]

decrease in the content of cellular ATP and acts as a non specific inhibitor of protein kinase activity. These effects might account for some of the metabolic actions of diamide in other cellular systems. Our findings in cultured fibroblasts are in agreement with reports showing that diamide inhibited both cAMP-dependant and -independant autophosphorylation of erythrocyte membrane by perturbing the protein substrates [17], that this compound interferes with TSH action in thyroid slices by inhibition of basal and TSH-stimulated cAMP production [18], and that it affects the permeability of adipose cells [19]. All these observations indicate that diamide does not provide a selective probe to define which metabolic processes are regulated by changes in protein kinase activity.

Acknowledgements

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