

# Induction of cell surface blebbing by increased cellular $P_i$ concentration

Mads MARCUSSEN

Department of Medical Biochemistry and Genetics, Biochemistry Laboratory B, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

Cell surface blebbing is an early, reversible characteristic of anoxia described in several different cell types. Blebbing may lead to the formation of large terminal blebs, and ultimately cell death upon rupture of the membrane. In the present work, evidence is presented indicating that  $P_i$  may be the immediate mediator of cell surface blebbing: (1) cell surface blebbing can be induced in normoxic Ehrlich ascites tumour cells by a high extracellular concentration of  $P_i$ , leading to an increase in the cellular  $P_i$  concentration; (2) anoxia induces sustained elevation

of the cellular  $P_i$  concentration and (3) cell surface blebbing during anoxia is reversed upon reoxygenation, and the disappearance of blebbing depends on the decrease in cellular  $P_i$  concentration. The rate of disappearance of blebs may be enhanced by the simultaneous addition of adenine and inosine to the growth medium. This leads to a decrease in cellular  $P_i$  concentration and to an almost complete restoration of the cellular ATP concentration. It is suggested that  $P_i$  is an important mediator of anoxia induced cell damage.

## INTRODUCTION

Cell surface blebbing has been demonstrated in several different cell types exposed to anoxia/ischaemia, including cardiac myocytes, freshly dissociated hippocampal neurons, hepatocytes, ascites tumour cells, and *in vivo* in endothelial cells from myocardial capillaries [1–6]. It is thought to be an important step in the formation of irreversible damage of the plasma membrane during anoxia.

Elevated cellular  $P_i$  content has been described *in vivo* in ischaemic brain tissue from patients suffering cerebral thrombosis [7], as well as in experimental ischaemic animal models [8,9] using  $^{31}\text{P}$ -NMR spectroscopy. Other characteristic features of anoxia are decreased cellular ATP content, metabolic acidosis, and, after prolonged  $\text{O}_2$  deprivation, a decrease in the total adenine nucleotide content (AMP + ADP + ATP). A decrease in the total adenine nucleotide content limits the capacity for ATP re-synthesis, and thereby normalization of cell function.

Cell surface blebbing has been suggested to be associated with changes in the dynamics of actin filaments observed during anoxia, resulting in conversion of G-actin to F-actin and thereby an increase in the F-actin:G-actin ratio [4,10]. This may be explained by an effect of  $P_i$  on the stability of the cytoskeleton, since the polymerization of both purified actin and tubulin is affected by the  $P_i$  concentration. The effect on actin polymerization is suggested to be mediated through association of  $P_i$  ( $\text{H}_2\text{PO}_4^-$  species) with F-ADP-actin. This affects the dynamics of the actin polymer because of the relative slow release of the F-ADP- $P_i$  subunits, compared with F-ADP subunits, from actin polymers [11]. This effect may explain the increased conversion of G-actin into F-actin described during chemical hypoxia [10]. Similarly, association of  $P_i$  with GDP bound to the E-site on tubulin affects the release of tubulin monomers from microtubules, leading to an increase in the polymer:monomer ratio [12]. Furthermore, it is well known that association of proteins (microtubule associated/actin associated proteins) to both actin- and tubulin-polymers may be regulated by protein phosphorylation. Increased cellular  $P_i$  concentration may lead to inhibition of protein dephosphorylation, and thereby a change in cytoskeletal dynamics [13].

It is highly feasible that perturbations in the dynamics of the

cytoskeleton mediated by  $P_i$ , either through a direct effect on the actin/tubulin monomers, and/or through actin/tubulin-associated proteins may lead to local weakening of plasma membrane-cytoskeleton interactions and, subsequently, cell surface blebbing. This possibility is supported by the present results.

## Materials and Methods

Silicone oil (density 1.05 g/ml) was obtained from Sigma, highly liquid paraffin from Merck (Germany), and  $^3\text{H}_2\text{O}$  and [*methoxy*- $^{14}\text{C}$ ]inulin from NEN (Germany).

Ehrlich cells, strain ELT, were maintained in female mice (NMRI) by intraperitoneal growth, and harvested 7 days after injection. Balb/c 3T3 cells were grown with standard procedures in Dulbecco's modified minimal essential medium supplemented with 10% (v/v) newborn calf serum.

Anoxia (Ehrlich cells) was obtained by incubating cells in pure nitrogen. Buffers for anaerobic experiments contained 12 mM Na-Hepes and 2 mM  $\text{NaPO}_4$  (at the pH indicated) 0.6 mM  $\text{MgCl}_2$ , 140 mM NaCl, 5 mM KCl and 2  $\mu\text{Ci/ml}$   $^3\text{H}_2\text{O}$ , unless otherwise stated. Aerobic experiments were performed at an extracellular pH [pH(o)] of 6.5 and with sodium phosphate at the concentration indicated. Cells were incubated at about  $10^7$  cells/ml, at 37 °C, and kept in suspension by gentle shaking. At the indicated times, aliquots were transferred to Eppendorf tubes on ice containing 0.4 ml of silicone oil/liquid paraffin mixture (final density 1.034 mg/ml) and centrifuged for 1 min at 20000 g. After centrifugation the tubes were washed four times with water before the oil layer was aspirated, and the cell pellet was extracted with 0.4 M perchloric acid (PCA), and a fraction thereof was neutralized with KOH.

From the PCA extract, ATP, ADP, AMP,  $P_i$  and cell volume were determined as previously described [14]. Shortly afterwards, nucleotides were separated and quantified by HPLC using a strong anion-exchange column (Partisil-10 Sax 2 mm  $\times$  200 mm, Whatman, Clifton, NJ, U.S.A.). Approx.  $10^6$  cells were required for  $P_i$  determination; neutralized PCA extract was mixed with acidic molybdate and Malachite Green and  $P_i$  content was quantified at 650 nm in a spectrophotometer [15]. Cellular water content was determined from the  $^3\text{H}_2\text{O}$  content of the medium

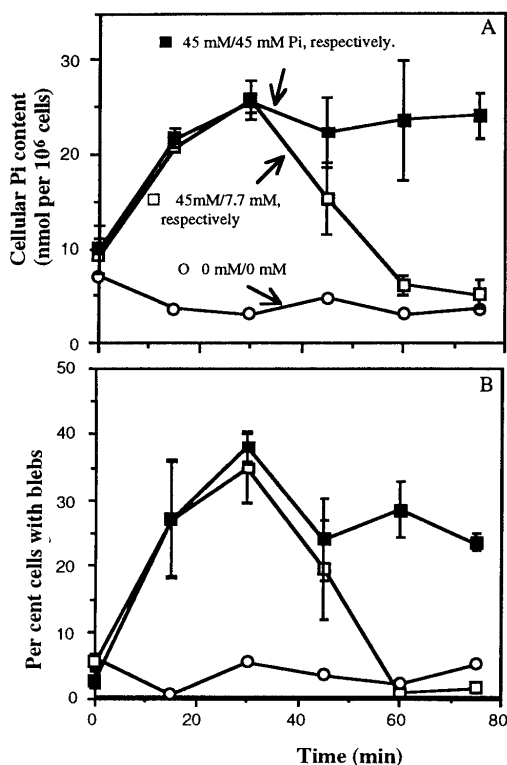
and that of the cells, which were isolated by centrifugation through oil; corrections were made for extracellular water based on experiments with [*methoxy*- $^{14}\text{C}$ ]inulin. Intracellular pH [ $\text{pH}(i)$ ] was determined from the transmembrane distribution of 5,5'-dimethyl[2- $^{14}\text{C}$ ]oxazolidine-2,4'-dione (DMO). DMO was added to the cell suspension at a final concentration of  $1.7\ \mu\text{M}$ ,  $0.1\ \mu\text{Ci/ml}$ . At the end of  $\text{pH}(i)$  experiments, the cell suspension was centrifuged through an oil layer composed of three parts corn oil and ten parts dibutylphthalate; cells were washed and extracted as described above.

Cells for microscopy were pelleted, resuspended in 4% (v/w) paraformaldehyde, fixed for 30 min, and resuspended in PBS, in which they could be stored for several days. Cell surface blebbing was determined in a phase-contrast microscope, counting 75–150 cells per experiment. Cell viability was determined in a parallel experiment, using Trypan Blue exclusion; less than 9% of cells were stained in all experiments. All data represent the average of at least four experiments  $\pm$  S.E.M.

## RESULTS AND DISCUSSION

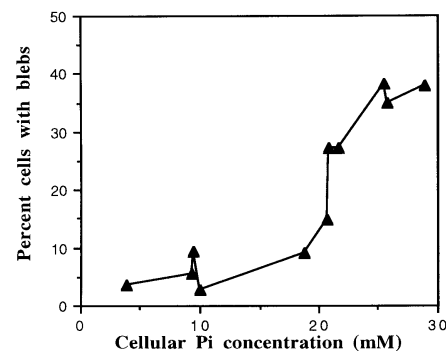
### Experiments in normoxic cells at high extracellular $\text{P}_i$ concentration and low pH

Cell surface blebbing may be induced by elevating the cellular  $\text{P}_i$  concentration in normoxic Balb/c 3T3 cells and Ehrlich cells with a normal ATP to ADP ratio. At high extracellular  $\text{P}_i$



**Figure 1** Time-dependent effect of increased extracellular concentration of  $\text{P}_i$  on cellular  $\text{P}_i$  concentration and bleb formation in Ehrlich cells under aerobic conditions

For about 30 min cells were incubated in either 45 mM (■, □) or 0 mM (○) extracellular  $\text{P}_i$ , pH 6.5. The 45 mM  $\text{P}_i$  medium was then changed to media containing either 45 mM  $\text{P}_i$  (■) or 7.7 mM  $\text{P}_i$  (□), pH 6.5. (A) Cellular  $\text{P}_i$  content in nmol/ $10^6$  cells. (B) Percentage of cells with one or more blebs, as determined by phase-contrast microscopy; non-viable cells were excluded. Values given  $\pm$  S.E.M. ( $n = 4$ ).



**Figure 2** Concentration-dependent effect of cellular  $\text{P}_i$  on the relative number of cells with blebs in normoxic Ehrlich cells

Cells were incubated for 40 min at  $\text{pH}(o)$  6.5 at different extracellular  $\text{P}_i$  concentrations ranging from 0 mM to 55 mM.

concentration (45 mM) a large increase in cellular  $\text{P}_i$  concentration may be obtained through ion-exchange of extracellular  $\text{H}_2\text{PO}_4$  with cellular  $\text{Cl}^-$ ; this process is highly dependent on the pH of the medium ([14,16,17] and M. Marcussen, K. Overgaard-Hansen and H. Klenow, unpublished work). At pH 6.5, which is optimal for the accumulation of  $\text{P}_i$ , this event is accompanied by bleb formation, as demonstrated in Figure 1 for Ehrlich cells. The kinetics of formation of cell surface blebbing closely resembles the kinetics of accumulation of cellular  $\text{P}_i$ , as clearly seen by comparing panels A and B of Figure 1. Upon removal of extracellular  $\text{P}_i$ , thereby decreasing the intracellular  $\text{P}_i$  content, cell surface blebbing is reversed to control levels. The ATP to ADP ratio remained constant during the whole observation period ( $10.9 \pm 0.9$ ), as well as the AMP concentration (cellular AMP concentration  $< 0.60$  mM).

The accumulation of  $\text{P}_i$  does not affect the  $\text{pH}(i)$ ; after 30 min the pH has decreased from 7.10 to 6.85 in the presence, and to 6.80 in the absence, of  $\text{P}_i$  at  $\text{pH}(o)$  6.5. Therefore it is unlikely that low  $\text{pH}(i)$  alone would be involved in the formation of cell surface blebbing.

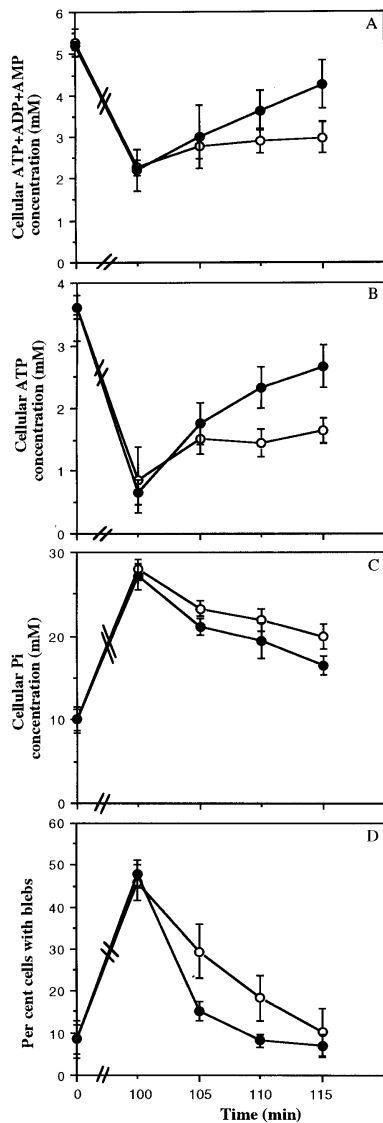
Blebbing may be induced also in normoxic, cultured Balb/c 3T3 cells by increasing the cellular  $\text{P}_i$  concentration, however, the rate of formation of blebs is significantly slower compared with Ehrlich cells, but incubation for 1 h in 45 mM extracellular  $\text{P}_i$  at pH 6.5 induces clearly visible blebbing (results not shown).

### Experiments in normoxic cells: effect of different cellular $\text{P}_i$ concentrations

In Figure 2 normoxic Ehrlich cells have been exposed to different extracellular  $\text{P}_i$  concentrations, ranging from 10 mM to 55 mM at  $\text{pH}(o)$  6.5. This results in cellular  $\text{P}_i$  concentrations ranging from 4 mM to 28 mM. It can be seen, that an increase in the intracellular  $\text{P}_i$  concentration up to approx. 20 mM does not induce changes in the relative number of cells with blebs. However, at cellular  $\text{P}_i$  concentrations exceeding 20 mM this number increases dramatically, and at 28 mM approx. 38% of the cells exhibit visible cell surface blebbing.

### Experiments in anoxic cells: effect of adenine and inosine on cell surface blebbing and cellular concentrations of adenine nucleotides and $\text{P}_i$

Increased cellular  $\text{P}_i$  concentrations of the same magnitude as described above may also be obtained by exposing cells to



**Figure 3** Effect of re-oxygenation of a suspension of Ehrlich cells after anoxia for 100 min

pH(o) was 7.4, extracellular  $P_i$  concentration was 2 mM. Re-oxygenation was in the absence (○), or in the presence (●) of inosine and adenine (100  $\mu$ M each). Effect on intracellular concentration of (A) adenine nucleotides (ATP + ADP + AMP) (mM); (B) ATP (mM); and (C)  $P_i$  (mM). (D) Percentage of cells with one or more blebs. Values given are  $\pm$  S.E.M. ( $n = 4$ ).

anoxia/hypoxia. In the following experiment Ehrlich cells have been exposed to anoxia for 100 min, and then reoxygenated for 15 min in the presence or absence of adenine and inosine (100  $\mu$ M each). At 100 min the intracellular concentration of  $P_i$  has increased from the normal value of about 7 mM to  $27.5 \pm 1.6$  mM (S.E.M.,  $n = 4$ ) (Figure 3C); a concentration that is similar to that obtained in normoxic Ehrlich cells at high extracellular concentration of  $P_i$  (see Figure 2). Also, the ratio of cells with blebs has increased in the same period, up to about 45%, which is comparable to the 36% obtained in normoxic cells at a cellular  $P_i$  concentration of 27 mM. These results indicate that cellular  $P_i$  concentration above approx. 20 mM is accompanied by cell surface blebbing, regardless of the way the  $P_i$  concentration

increase is obtained, i.e. either through anoxia or during normoxia with a high extracellular  $P_i$  concentration at low pH.

During anoxia ATP is partially degraded to adenosine and  $P_i$  via AMP, and further to inosine and hypoxanthine. The  $P_i$  released from ATP contributes to the increase in cellular  $P_i$  concentration during anoxia; however, other phosphorylated compounds may also contribute, e.g. GTP, NAD, NADP and phosphocreatine. Upon reoxygenation, inosine formed during anoxia may serve as an energy source supporting the conversion of AMP and ADP to ATP, but the adenine nucleotide pool (ATP + ADP + AMP), the ATP pool, and the low cellular concentration of  $P_i$  cannot be completely restored. This is clearly indicated by Figure 3(B), where the ATP concentration of the cells that have not been treated with adenine and inosine (open symbols) reaches only approx. 50% of that of the control at 0 min. The formation of ATP is accompanied by a decrease in cellular  $P_i$  concentration and a decrease in the relative number of cells with blebs (Figures 3C and 3D respectively). Addition of inosine to the growth medium may result in the formation of ribose-1-phosphate, which may be converted into 5-phosphorylribose-1-pyrophosphate (PRPP) via ribose-5-phosphate. This process is stimulated by a high  $P_i$  concentration, as demonstrated in normoxic erythrocytes, where the PRPP content may be increased up to 1000-fold [18,19]. The PRPP formed in this way can be utilized by added adenine to form AMP. In this way the adenine nucleotide pool may be restored independently of *de novo* purine synthesis, resulting in a further decrease in cellular  $P_i$  when compared with the untreated controls. This is closely followed by a decrease in the ratio of cells with blebs compared with the untreated controls. That the  $P_i$  and ATP levels may be almost completely restored in this way (Figures 3C and 3B respectively) indicates that sufficient PRPP is indeed formed in this system to support the formation of ATP from adenine, despite an initially low ATP concentration. Adenine added alone without inosine is not converted into ATP (results not shown). It is concluded that the decrease in  $P_i$  concentration after reoxygenation is accompanied by a decrease in the number of cells with blebs (Figures 3C and 3D respectively), and that the rate of decrease in both blebbing and  $P_i$  concentration may be enhanced by the addition of adenine and inosine to the growth medium during the period of reoxygenation. It can also be seen that a relatively small decrease in the  $P_i$  level is needed to reverse blebbing, i.e. from approx. 27 mM to 20 mM, compared with the normal level of about 7 mM.

#### Effect of pH(o) on cellular $P_i$ concentration and cell surface blebbing during anoxia

The large increase in cellular  $P_i$  concentration during anoxia may, in principle, be a result of excessive uptake of phosphate from the growth medium and/or trapping of cellular phosphate released from phosphorylated compounds. Omission of phosphate from the growth medium does not, in this experimental set-up, affect  $P_i$  accumulation during anoxia (results not shown), indicating that the increase in intracellular  $P_i$  is not, under these conditions, derived from the growth medium. This fact may be explained by the relatively high cellular ATP concentration, about 0.75 mM, observed after 100 min of anoxia.  $P_i$  accumulation is, however, affected by the pH(o), as indicated in Table 1. At both pH 6.0 and pH 7.4 the cellular  $P_i$  concentration increases from the normal level, but the increase at pH 7.4 exceeds that at pH 6.0 by 5.8 mM, corresponding to a difference in the increase in cellular  $P_i$  concentration of 28%, despite the fact that ATP degradation at pH 6.0 equals that at pH 7.4. This difference in  $P_i$  accumulation is reflected in the percentage of cells with blebs

**Table 1** Effect of anoxia on the cellular  $P_i$  concentration and bleb formation in Ehrlich cells at pH 6.0, and pH 7.4 respectivelyAnoxia was applied by superfusing the incubation bottles with  $N_2$  for 60 min.

	Extracellular pH ...	
	6.0	7.4
Cellular $P_i$ concentration (mM)	20.5 ± 1.5	26.3 ± 1.5
Ratio of cells with blebs (%)	22 ± 2.5	42 ± 10.5
Cellular ATP concentration (mM)	0.4 ± 0.3	1.1 ± 0.6

after 60 min, as indicated in the Table. Furthermore, the numbers in the Table correlate very well with the relationship between cellular  $P_i$  concentration and cell surface blebbing in normoxic cells demonstrated in Figure 2.

This effect of pH(o) on the accumulation of cellular  $P_i$  during anoxia could be explained by either a stimulating effect of  $H^+$  on phosphate-chloride exchange, as previously described for the reverse process ([14] and M. Marcussen, K. Overgaard-Hansen and H. Klenow, unpublished work), or by inhibition of sodium-phosphate co-transport by  $H^+$  [16], or a combination of the two transport mechanisms. The large concentration gradient of  $P_i$  between the extracellular, and the intracellular space may thus be sustained by salvage of intracellular  $P_i$  obtained by sodium-phosphate co-transport, driven by the passive sodium influx during anoxia. At acidic pH, e.g. 6.0, phosphate-chloride exchange may tend to inhibit the increase in cellular  $P_i$  concentration. Substituting extracellular sodium with the non-permeable cation choline during anoxia does not affect either bleb formation or  $P_i$  accumulation in Ehrlich cells (results not shown), indicating that a sodium-independent phosphate transport mechanism may compensate for a possible sodium-phosphate co-transport in the absence of sodium. However, in freshly dissociated hippocampal neurons, exchanging extracellular  $Na^+$  with the non-permeable cation *N*-methyl-D-glucamine (NMDG<sup>+</sup>) has a clear effect on bleb formation during and after anoxia [2].

In conclusion, the present work clearly shows that cellular  $P_i$  concentration may be elevated, either by increasing the extracellular  $P_i$  concentration, or by exposing cells to anaerobic conditions. Furthermore, by comparing Figure 2 and Table 1 it can be seen that a cellular  $P_i$  concentration of approx. 26 mM, obtained either through anoxia, or through increased extracellular  $P_i$  concentration, results in comparable ratios of cells with cell surface blebbing, 42% and 35% respectively. Thus a decreased ATP concentration during anoxia does not seem to be involved in bleb formation, since elevation of the extracellular  $P_i$  concentration under normoxic conditions does not affect the cellular ATP concentration. The 'pH-paradox' [1,10], referring to the protective effect of acidic pH during, and after, anoxia on alterations in cytoskeleton dynamics (G-actin:F-actin ratio) and on bleb formation, may be explained by an inhibitory effect of  $H^+$  on  $Na^+/H_2PO_4^-$  co-transport (M. Marcussen, K. Overgaard-

Hansen and H. Klenow, unpublished work), and/or by a stimulating effect of  $H^+$  on the loss of  $P_i$  due to ion exchange with extracellular  $Cl^{*-}$ , as shown for the reverse process [14], resulting in a lower cellular  $P_i$  concentration at acidic pH (Table 1). A difference of 5.8 mM in cellular  $P_i$  concentrations at pH 6.0 and pH 7.4 respectively may very well explain the observed difference in the ratio of cells with blebs (see Figure 2). The following sequence of events leading to cell surface bleb formation during anoxia is suggested. (1) ATP degradation during  $O_2$  deprivation and release of  $P_i$ . (2) Inability of the  $Na^+/K^+$  ATPase to maintain the normal ionic gradients across the plasma membrane, resulting in trapping of  $P_i$  released from phosphorylated cellular compounds, primarily ATP, leading to an increase in cellular  $P_i$  concentration. (3) A decrease in the G-actin:F-actin ratio due to a low  $K_d$  of F-actin- $P_i$  present at high  $P_i$  concentrations compared with the  $K_d$  of F-actin. A similar effect applies to the  $K_d$  of tubulin polymers containing GDP- $P_i$  compared with those containing GDP at the E-site of tubulin. (4) Disturbance of plasma membrane-cytoskeleton interaction. (5) Cell surface blebbing. Furthermore, the present results show that simultaneous administration of adenine and inosine during reoxygenation leads to an almost complete recovery of the normal level of ATP and substantial reduction of the  $P_i$  increase within 15 min. It is suggested that adenine and inosine administered together may be used in the treatment of ischaemia-induced tissue damage.

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