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cAMP Activates Na^+/H^+ Antiporter in Murine Macrophages

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Summary: The role of cAMP in activating the Na^+/H^+ antiporter in murine macrophage (MØ) system was investigated. Incubation of PU5-1.8 macrophage tumour cells, peritoneal MØ and bone marrow derived macrophages (BMDMØs) with dibutyryl-cAMP (db-cAMP) or cholera toxin (CT) led to an increase in intracellular pH (pH_i). The magnitudes of these responses differed markedly in the three cell types, BMDMØs being the most sensitive, PU5-1.8 cells the least so. These cells also differed in their responses to inhibitors of Na^+/H^+ exchange. In PU5-1.8 cells, the db-cAMP- or CT-triggered intracellular alkalinization was abolished by amiloride treatment which, however, was ineffective in BMDMØs. The chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP), also caused a significant increase in cytoplasmic pH. However, its action was apparently not mediated by cAMP. The significance of these observations is discussed.

The maintenance of an intracellular pH within narrow limits is a prerequisite and feature of living cells. It was demonstrated that an electroneutral, amiloride-sensitive Na^+/H^+ antiport system plays a central role in this homeostasis (1). The changes in intracellular pH (pH_i) resulting from the activation of Na^+/H^+ antiporter also appears to affect a broad spectrum of cellular functions including cell volume regulation (2), DNA synthesis (3), and differentiation of pre-B lymphocytes (4). The mode of activation of the Na^+/H^+ exchanger has been recently reviewed (5,6). It is generally accepted that the rate of Na^+/H^+ antiport is reduced by elevating the cytoplasmic level of cAMP (6). Examples are found in lymphocytes (7), epithelial cells (8),

Abbreviations used are: BMDMØ, bone marrow-derived macrophage; BCECF, 2',7'-bis(carboxylethyl) 5,6-carboxyl-fluorescein; CSF-1, colony stimulating factor-1; CT, cholera toxin; db-cAMP. dibutyryl cAMP; FCS, fetal calf serum; FMLP, N-formyl-methionyl-leucyl-phenylalanine; PKC, protein kinase C.

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renal brush border vesicles (9) and cells from small intestine (10). Recent data also demonstrated that Na^+/H^+ antiport activity in reconstituted proteoliposomes was inhibited by protein phosphorylation which required a cAMP-dependent protein kinase (11). By contrast, it was observed in fish erythrocytes that stimulation of the Na^+/H^+ antiporter is cAMP-dependent (12). In light of this, the role of cAMP in activation of Na^+/H^+ antiporter in murine macrophage system was studied. Here, it was demonstrated that elevation of intracellular cAMP level stimulates Na^+/H^+ antiport activity.

Materials and Methods

Materials: PU5-1.8 cells were derived from Balb/c mice macrophage tumour. Fetal calf serum (FCS) was obtained from Gibco and the 2',7'-bis(carboxyl-ethyl)-5,6-carboxylfluorescein-acetoxylmethyl ester (BCECF-AM) was purchased from Calbiochem. All other reagents were from Sigma.

Cell culture: PU5-1.8 cells were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in a 5% CO_2 incubator. Cells grown to confluence were arrested at Go phase by a 24-hour incubation in serum-free medium.

Peritoneal macrophages were obtained from peritoneal lavage with sterile phosphate buffer saline (PBS). After washing, cells were resuspended in serum-free RPMI 1640 medium.

BMDMØs were established as reported (13). Briefly, normal femoral bone marrow stem cells from 6 week-old Balb/c mice were flushed into RPMI 1640 medium which contained 10% FCS and 20% (v/v) L-cell conditioned medium as a source of CSF-1. Cell suspension was placed in a 24-well plate and incubated for 7 days at 37°C, 5% CO_2 incubator.

Measurement of cytoplasmic pH: Cytoplasmic pH was determined by the BCECF-AM fluorescent probe as described (14) with excitation wavelength 505nm and emission wavelength 530nm. Cells were loaded with BCECF-AM (2μM final concentration) for 30 mins at 37°C. After washing, the fluorescence was determined in a HEPES buffer medium (140mM NaCl, 5mM KCL, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 10mM glucose, pH7.1). Fluorescence was calibrated against pH after cell disruption with 0.5% triton X-100.

Results and Discussion

The existence of Na^+/H^+ antiporters on the cell surface of murine macrophage cell line PU5-1.8 was examined. As shown in Fig.1, addition of 10μM FMLP to growth-arrested PU5-1.8 cells elicited an intracellular alkalinization, up to 0.1 pH unit above the initial steady-state pH_i. In most cases, there was a lag period of 1 to 1.5 min, and the pH_i was maintained at the elevated state for several minutes (Fig.1a). The increase in pH_i was completely

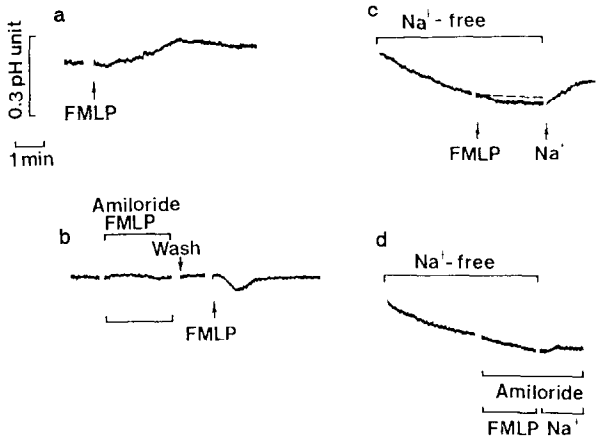

Fig.1 FMLP Induced pH_i Changes in PU5-1.8 Cells.
PU5-1.8 cells were loaded with BCECF; cellular fluorescence was measured at 37°C as described. Cell treatments were as shown in figure. Where indicated, 1mM amiloride, 10μM FMLP or 140mM Na^+ was added. In (c) and (d), $choline^+$ replaced Na^+ iso-osmotically in Na^+-free buffer.

inhibited by 1mM amiloride (Fig.1b). After washing the cells to remove amiloride, re-addition of 10μM FMLP resulted in a biphasic response, comprising an intracellular acidification followed by alkalinization which caused the pH_i to recover the resting value subsequently. This sensitivity to amiloride indicates that the pH effect of FMLP was probably mediated by the well-known Na^+/H^+ antiporter. Corroborative evidence was obtained with the use of Na^+-free buffer in which Na^+ had been iso-osmotically replaced by $choline^+$. In this system, BCECF-loaded PU5-1.8 cells showed a steady decrease in intracellular pH (Fig.1c) presumably due to an influx of H^+ through the reversed operation of the Na^+/H^+ antiporter (15). Exposure of cells to 10μM FMLP in this Na^+-free medium failed to stem the cytosolic acidification. However, readdition of Na^+ caused an immediate and rapid recovery of pH_i. This recovery was not evident when amiloride was used to inhibit the Na^+/H^+ antiporter (Fig.1d). Similar results were obtained in BMDMØs (data not shown).

It is generally thought that in mammalian cells cAMP acts as a modulator to dampen the activity of the Na^+/H^+ antiporter (6-10). However, as shown in Fig.2a, addition of db-cAMP alone to resting PU5-1.8 cells increased cytosolic pH (pH_i). The rise of pH_i was largely inhibited by pre-treating the cells with 1mM amiloride for 5 mins, thereby confirming the involvement of the

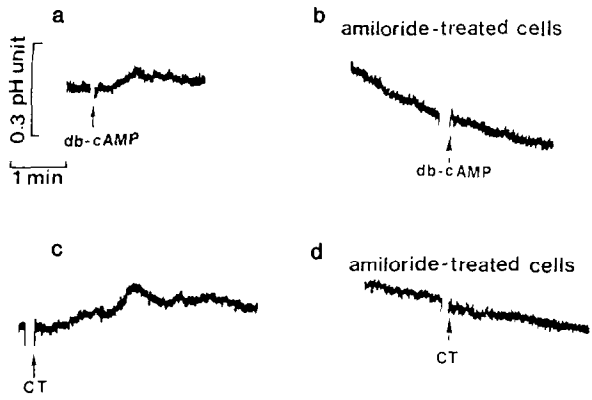

Fig.2 Role of db-cAMP and cholera toxin on the pH_i in PU5-1.8 cells.
PU5-1.8 cells were loaded with BCECF as described in the experimental procedure for fluorescence determination. Where required, cells were incubated with 1mM amiloride for 5 mins before use. Other additions were: db-cAMP (5µg/ml), CT (100ng/ml). Traces are representative of at least 3 experiments.

Na^+/H^+ antiporter in the alkalinization process (Fig.2b). Similarly, incubation of PU5-1.8 cells with 100ng/ml cholera toxin (CT), a potent cAMP elevating agent, also elicited an increase of pH_i (Fig. 2c). This effect of CT was again inhibited in the presence of amiloride (Fig. 2d). These results suggest that cAMP stimulates the amiloride-sensitive Na^+/H^+ antiporter in PU5-1.8 cells, in contrast to its effect in other mammalian cells in a bicarbonate-free system (6).

As shown in Fig.3, FMLP induced an intracellular alkalinization (Fig.3a) and it is interesting to note that cholera toxin, while itself stimulatory of Na^+/H^+ antiport, did not prevent the effect

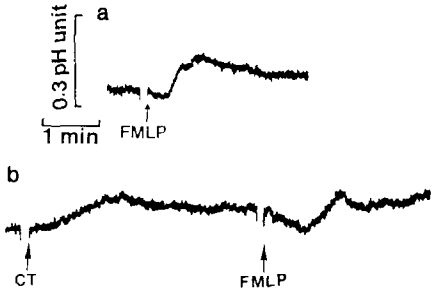

Fig.3 Role of db-cAMP and cholera toxin on the pH_i in PU5-1.8 cells.
PU5-1.8 cells were loaded with BCECF as described. Where indicated, 5µg/ml db-cAMP and 10µM FMLP (a) or 100ng/ml CT with 10µM FMLP (b) were added. Traces are representative of at least 3 experiments.

Table I. Effect of FMLP on cAMP level in PU5-1.8 cells

Treatment	[cAMP] (pmole/ 10^6 cells)[a]
Control	1.1 ± 0.2
FMLP (0.1 μM)	0.9 ± 0.2
FMLP (1 μM)	0.9 ± 0.05
FMLP (10 μM)	1.1 ± 0.1
FMLP (100 μM)	1.1 ± 0.1
Cholera toxin (100ng/ml)	4.3 ± 0.2

[a]Cells (1 x 10^6/sample) were pre-treated with 1mM theophylline for 45 mins. Cells were then treated with FMLP or CT for 30 mins. Cellular cAMP level was determined by radioimmuno-assay according to established procedures. Results are mean ± SD for 3 experiments.

of FMLP (Fig.3b). This observation suggests that messengers other than cAMP, most probably protein kinase C (PKC), might trigger Na^+/H^+ antiport activity. Partial corroboration for this hypothesis came from the observation that FMLP ranging from 0.1μM to 100μM did not raise intracellular cAMP level in theophylline-pretreated PU5-1.8 cells while CT (100ng/ml) occasioned a 4-fold increase (Table I).

Essentially similar results were observed with peritoneal macrophages (data not shown) and bone marrow derived macrophages although the magnitudes of the responses were much pronounced in these cell types than in PU5-1.8 cells. In BMDMØs, where the changes were the greatest, it is seen in Figs.4a and 4b that both db-cAMP and CT generated a sustained cytosolic alkalinization. Pre-incubation of cells with these agents again did not block the FMLP-induced cytoplasmic alkalinization (Figs.4a, 4b). Moreover, incubation of BMDMØs with 1mM amiloride for as long as 10 mins, in contrast to the effectual duration of 5 mins in peritoneal MØs and PU5-1.8 cells, failed to abolish the cytoplasmic alkalinization induced by db-cAMP and CT (Fig.4c). These observations are consistent with the suggestion that BMDMØs are relatively immature cells and therefore much more sensitive to proliferative stimulation than their more mature counterparts.

To date, at least 3 different mechanisms have been proposed to activate Na^+/H^+ antiport activity in a variety of cell types. In mouse NIE-115 neuroblastoma cells, for example, stimulation of PKC results in the activation of Na^+/H^+ antiporter (16, 17). In human fibroblasts, there is evidence to show that activation of

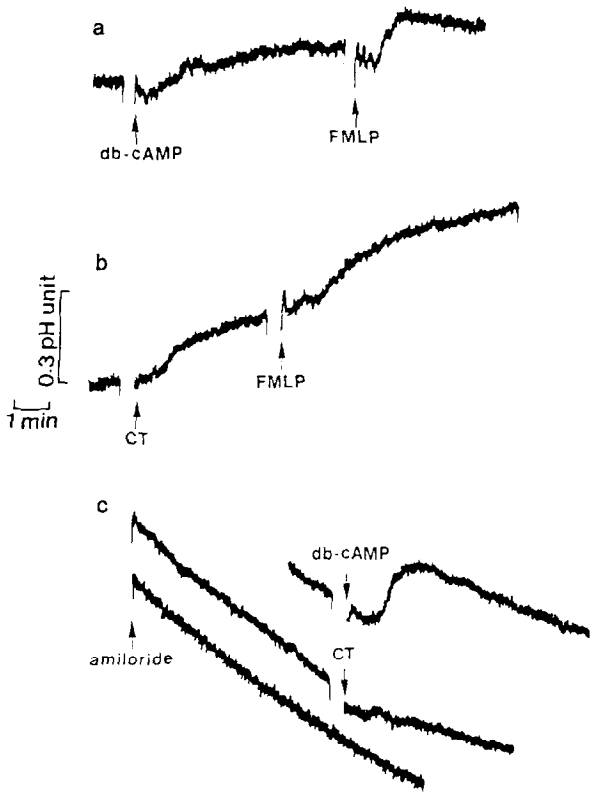

Fig.4 Role of db-cAMP and cholera toxin on the pH_i in bone marrow derived macrophages.
Bone marrow derived macrophages were loaded with BCECF as described. (a) 5µg/ml db-cAMP and 10µM FMLP or (b) 100ng/ml CT with 10µM FMLP were added. For (c), cells were pre-treated with 1mM amiloride for 10 mins before use. Traces are representative of at least 3 experiments.

Na^+/H^+ antiporter is mediated by an increase in cytoplasmic Ca^{2+} (16). Recently, Na^+/H^+ exchange has been reported to be stimulated by α_2-adrenergic agonists (18). These observations serve to illustrate the multiplicity of control exerted on Na^+/H^+ antiport activities. The multiplicity is apparent not only in antiporters from different tissues but also in the same cell. We demonstrate here that cAMP serves to stimulate the antiporter in murine MØs although it has been shown to act as a negative modulator in other cell systems. Furthermore, the Na^+/H^+ antiporter, more apparent in BMDMØs but less so in the more mature PU5-1.8 cells, is regulated by at least two signals, of which cAMP is but one. Experiments are in progress to identify the mechanism whereby FMLP triggers the Na^+/H^+ antiport activity in murine macrophage systems.

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