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Relationship between intracellular pH changes, activation of protein kinase C and NADPH oxidase in macrophages

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Activation of the superoxide-generating NADPH oxidase by phorbol ester or zymosan induced a cytoplasmic acidification when liver macrophages were incubated in sodium-free media or in the presence of amiloride. Staurosporine or desensitization of protein kinase C inhibited phorbol ester- and zymosan-induced pH changes and generation of superoxide. The intracellular pH remained unchanged in cells incubated in physiological sodium media. Ionomycin and arachidonic acid did not induce a change in intracellular pH or a generation of superoxide. Fluoride, which has been shown to induce a translocation of protein kinase C in these cells, did not elicit superoxide generation but induced a decrease in intracellular pH. These experiments support (1) a role of the Na^+/H^+ antiporter in macrophages as a metabolic regulator of intracellular pH upon stimulation of the superoxide-generating NADPH oxidase, and (2) suggest an involvement of protein kinase C in this process.

Cellular pH; Superoxide; NADPH oxidase, Na^+/H^+ antiporter; Protein kinase C; Macrophage

1. INTRODUCTION

An antiport that exchanges Na^+ for internal H^+ plays an essential role in the regulation of cytoplasmic pH (pH_i) in most mammalian cells [1–3]. The Na^+/H^+ antiporter has been suggested to be stimulated directly by e.g. protein kinase (PK) C and/or by cytoplasmic acidification [1–3]. In macrophages an increased metabolic acid production occurs e.g. during activation of the superoxide-generating NADPH oxidase [4,5]. The mechanism of this process and its topology necessarily leads to an accumulation of H^+ in the cytosol since one proton is generated in the cytosol for every O_2^- appearing in the extracellular space [4,5]. Recently, we could show that inhibition of the Na^+/H^+ antiporter or artificial intracellular acidification of liver macrophages leads to an inhibition of prostanoid synthesis but has no effect on the generation of superoxide [6]. We interpreted these data as a differential effect of pH_i on phospholipase A_2 and NADPH oxidase, the key enzymes of these two cellular pathways. The present study was undertaken to investigate the relationship among generation of superoxide, activation of PKC and pH_i in cultured liver macrophages.

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2. EXPERIMENTAL PROCEDURES

2.1. *Materials*

Phorbol 12-myristate 13-acetate (PMA) was from Pharmacia, Freiburg (Germany). Arachidonic acid, cytochrome C, nigericin, 4α-phorbol 12,13-didecanoate, staurosporine and zymosan were purchased from Sigma, Munich, (Germany). 2',7'-bis(carboxyethyl)-5(6')-carboxyfluorescein (BCECF), its pentaacetoxymethyl ester (BCECF-am) and ionomycin were obtained from Calbiochem, Giessen (Germany). Amiloride hydrochloride dihydrate was a gift of Merck Sharp and Dohme Research Laboratories (Rahway, USA). Leighton tubes were purchased from Tecnomara, Fernwald (Germany). All other chemicals were of analytical grade.

2.2. *Cell culture*

Liver macrophages were isolated and cultured as described previously [7].

2.3. *Determination of pH_i*

Cells attached to Leighton tube slides were incubated in RPMI medium containing 1% newborn calf serum and 25 μM BCECF-am (added from a 2.5 mM stock solution in dimethyl sulphoxide) for 30 min at 37°C. Thereafter, the loaded cells were washed thoroughly with Hanks' solution (122 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.38 mM KH_2PO_4, 0.34 mM K_2HPO_4, 10 mM glucose, 20 mM HEPES, pH 7.4) and the Leighton tube slides were fixed with a special holder at a 45° angle in a thermostatted cuvette containing 3 ml Hanks' solution. Fluorescence measurements were performed with stirring at 30°C in a RF-5000 Shimadzu spectrofluorometer. The wavelength for excitation and emission were 504±1.5 nm, 440±1.5 nm and 527±1.5 nm, respectively. The nigericin/potassium method of Thomas et al. [8] was used for calibration of fluorescence versus pH_i. Briefly, the cells were incubated in Hanks' solution containing 122 mM KCl, 5.4 mM NaCl, 10 μM nigericin and the external pH adjusted to different pH values with HCl or KOH. At these conditions pH_i will equal pH_o [8]. The ratio of 504 nm/440 nm fluorescence recorded at various known pH_o (=pH_i) values yielded a calibration curve which was then used to estimate pH_i in resting and

stimulated cells. All the experiments were performed at 30°C. Leakage of dye during the time of measurement was less than 2% and could be neglected. It was estimated by measuring the fluorescence in the cuvette after removing the Leighton tube slide. Even distribution of fluorescence throughout the cytosol was ascertained by comparatative phase and fluorescence microscopy.

2.4. *Determination of superoxide*

Cells were routinely cultured on 24-well plates and the content of superoxide in cell media measured as superoxide dismutase-inhibitable cytochrome C reduction [6]. Experiments with cells cultured on Leighton tube slides gave the same results (data not shown).

2.5. *Determination of PKC activity*

Activity of PKC in subcellular fractions was determined as described previously. Specific activity is expressed as the difference between the values obtained in the presence and absence of Ca^{2+} and lipids, respectively [9,10].

3. RESULTS AND DISCUSSION

The classical approach for studying the presence and the role of the Na^+/H^+ antiporter in pH_i-regulating mechanisms is to perturb cells rapidly by loading them with H^+ and then to monitor the recovery towards the initial pH_i value in physiological sodium media without or with amiloride, a known inhibitor of the Na^+/H^+ antiporter [11], or in sodium-depleted media [1]. One largely used technique to achieve this goal is the ammonia prepulse technique [1]. When liver macrophages were loaded with NH_4Cl and then external ammonia is removed a rapid recovery towards the initial pH_i value (7.26±0.05) occurred in physiological sodium media (Fig. 1A). Recovery of pH_i was much slower in the presence of amiloride (Fig. 1C) or by incubating the cells in sodium-depleted media (Fig. 1B). These results demonstrate the presence and importance of the Na^+/H^+ antiporter in regulating pH_i in cultured liver macrophages.

Fig. 2 shows that addition of phorbol ester to liver macrophages caused a rapid intracellular acidification of 0.3–0.4 pH units when the experiments were performed in sodium-depleted media whereas no change in pH_i was observed at physiological concentrations of sodium. PMA is known to exert its action via translocation and activation of PKC [12] and in liver macrophages leads to an enhanced generation of eicosanoids and superoxide [6,9]. Activation of the superoxide-generating NADPH oxidase is accompanied by a production of H^+ in the cytosol and if these are not removed to an intracellular acidification [4,5]. The PMA-induced pH_i decrease could therefore be due to an inhibition of the Na^+/H^+ antiporter and/or to an activation of NADPH oxidase. To elucidate this question, the effect of different compounds on pH_i (Table I) and superoxide generation (Table II) were investigated.

In resting cells the steady-state pH_i was determined to be about 7.25 and was slightly lower when the cells were incubated in sodium-depleted media (Table I). This indicates the absence of a pronounced metabolic

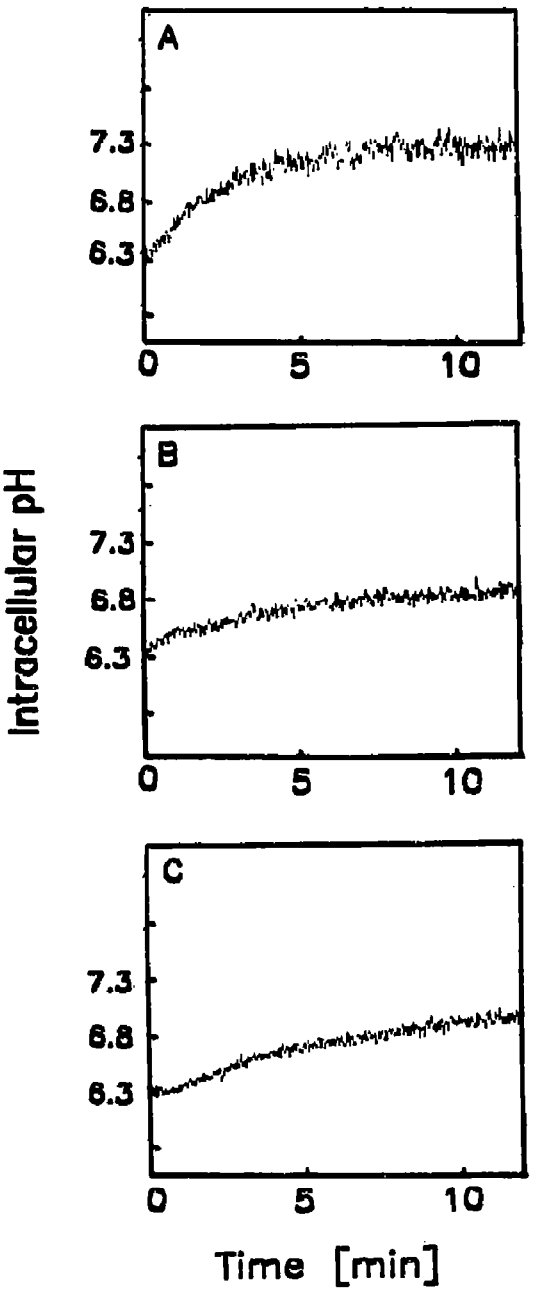

Fig. 1. Recovery of pH_i from NH_4Cl-preloaded liver macrophages. Liver macrophages (72 h in primary culture) were loaded with BCECF and pH_i was determined as described in section 2. The cells were first exposed to 20 mM NH_4Cl for 20 min; at time zero medium was changed and the cells incubated in Hanks' solution containing physiological concentrations of sodium without (A) and with (C) 200 μM amiloride or in sodium-depleted Hanks' solution ((B), NaCl was replaced iso-osmotically by *N*-methyl-D-glucamine-HCl). A typical set of data is shown which was reproduced at least four times.

acid production in unstimulated cells. The addition of PMA to cells incubated with amiloride or in sodium-depleted media induced a decrease of pH_i of 0.3–0.4 pH units whereas in physiological sodium-media pH_i remained unchanged. In platelets, peritoneal macrophages and some other cells PMA has been shown to elicit under appropriate conditions an intracellular ala-

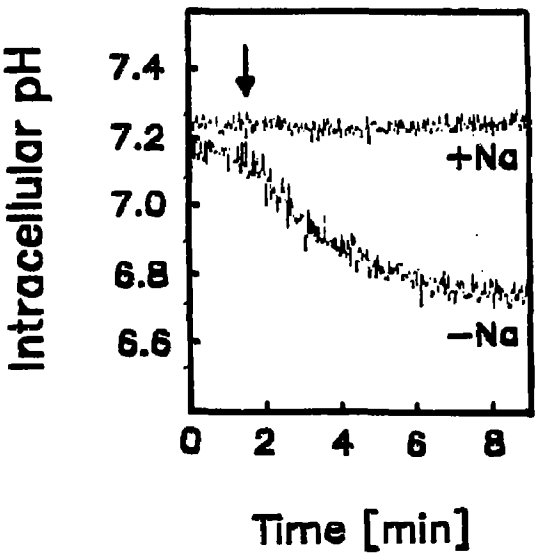

Fig. 2. Effect of PMA on pH_i in liver macrophages. Liver macrophages (72 h in primary culture) were loaded with BCECF and pH_i was determined as described in section 2. After defining the base-line pH_i, PMA (1 μM) was added as indicated by the arrow. +Na, Hanks' solution containing physiological sodium concentrations; −Na, sodium-depleted Hanks' solution (NaCl was replaced iso-osmotically by *N*-methyl-D-glucamine-HCl). A typical set of data is shown which was reproduced at least six times.

kalinization [1,13–15]. These results are often interpreted by the authors as a direct effect of PKC on Na^+/H^+ antiporter. The impotency of PMA to induce a similar alkalinization in liver macrophages (Fig. 1, Table I) may indicate that in these cells the Na^+/H^+ antiporter is not directly regulated by PKC or that activation of the Na^+/H^+ antiporter balances the acidification due to the activation of the NADPH oxidase. Furthermore, PMA elicited an enhanced generation of superoxide independently if the cells were treated with amiloride or incubated in sodium-depleted media (Table II, [6]). The effect of PMA on pH_i (Table I) and superoxide release (Table II) seems to be mediated by PKC sinde an inactive phorbol ester, 4α-phorbol 12,13-didecanoate [16], was without any effect and inhibition [17] or desensitization [9] of PKC with staurosporine or pretreatment with PMA, respectively, suppressed both responses. A regulatory role of PKC in this process is supported by the previous findings that PMA-induced superoxide production is also suppressed by K252a [9], RO 31-7549 and RO 31-8220 [18], three other inhibitors of PKC.

Besides PMA, zymosan induced a release of superoxide (Table II) and a decrease of pH_i (Table I) of about 0.4 pH units in sodium-depleted media. Both responses were suppressed by staurosporine or desensitization of PKC (data not shown) indicating that the effects of zymosan are also mediated by PKC.

Ionomycin and arachidonic acid had no effect on pH_i (Table I) and did not elicit a release of superoxide (Table II). However, these agents are known to induce

Table I

Effect of different agents on pH_i

The pH_i in liver macrophages (72 h in primary culture) was determined as described in section 2. In the Na^+-depleted medium (−Na) NaCl was replaced iso-osmotically by *N*-methyl-D-glucamine-HCl. After addition of PMA (1 μM), 4α-phorbol 12,13-didecanoate (1 μM), ionomycin (5 μM), arachidonic acid (30 μM) or potassium fluoride (30 mM) fluorescence was continuously recorded for 15 min. Amiloride (200 μM), staurosporine (1 μM) or PMA (100 nM) were added 10 min or 24 h prior to the stimuli, respectively. For the determination of pH_i in zymosan-stimulated cells, zymosan (0.5 mg/ml) was added to cells incubated in Leighton tubes for different periods of time and thereafter pH_i determined as described in section 2 (phagocytosis of zymosan was diminished in cells incubated in cuvettes and by magnetic stirring). Maximal pH_i changes were obtained 8 and 15 min after the addition of PMA and zymosan, respectively. Data are means±S.D. of three to six independent experiments and represent maximal pH_i changes. *P* values were calculated using Student's *t*-test for unpaired samples; $^*P \leq 0.006$.

Treatment	pH_i		Difference[a]
	+Na	−Na	
None	7.26±0.05	7.18±0.08	−0.08
PMA	7.21±0.08	6.83±0.02*	−0.38
+amiloride	6.88±0.04*	6.80±0.02*	−0.08 (−0.38)[b]
+staurosporine	7.26±0.07	7.20±0.04	−0.06
+PMA (24 h)	7.24±0.03	7.22±0.06	−0.02
4α-Phorbol 12,13-didecanoate	7.23±0.10	7.20±0.10	−0.03
Zymosan	7.27±0.03	6.88±0.05*	−0.39
Ionomycin	7.25±0.04	7.17±0.12	−0.08
Arachidonic acid	7.24±0.09	7.20±0.05	−0.04
Fluoride	7.23±0.08	6.88±0.09*	−0.35

[a](+Na) − (−Na); [b](+Na, −amiloride) − (−Na, +amiloride)

a formation of prostanoids in liver macrophages [10,19] suggesting that synthesis and release of prostanoids is not associated with changes in pH_i. The inability of ionomycin to induce a change in pH_i (Table I) suggest that in these cells cytosolic Ca^{2+} plays no central role in regulating pH_i. This assumption is confirmed by the findings that the effect of PMA and zymosan on pH_i (Table I) did not require the presence of extracellular Ca^{2+} (data not shown). These data are in contrast to results obtained with platelets, fibroblasts and some other cells [1,20–22] where cytosolic Ca^{2+} has been shown to be an important regulator of pH_i.

Fluoride induced in liver macrophages (incubated in sodium-depleted media) a decrease of pH_i (Table I) but not a generation of superoxide (Table II). Therefore, the fluoride-induced intracellular acidification can not arise from protons generated by NADPH oxidase. The mechanism of how fluoride induces a decrease of pH_i is not quite understood. It may be that fluoride induces an activation of other acid-forming processes leading to intracellular acidification or that the effect of fluoride is due to inhibition of protein phosphatases. On the other hand we recently showed [10] that fluoride leads to a translocation and activation of PKC in these cells

Table II

Effect of different agents on superoxide generation and translocation of PKC.

Superoxide generation in liver macrophages (72 h in primary culture) was determined as described in section 2. Experimental conditions were as described in legend of Table I. Data are means±S.D. of three to six independent experiments. Data for translocation of PKC are from [9,10]. Total activity in the homogenate was set to 100% and corresponds to 658±57 pmol/min × mg. Treatment of the cells with the different agents did not alter this activity. [a]Treatment with PMA (100 nM) for 24 h led to a complete disappearance of PKC in Kupffer cells [9]. n.d., not determined.

	Superoxide release (nmol/10^6 cells × 60 min)		PKC activity (% of control)	
Treatment	+Na	−Na	Soluble	Particulate
None	5±3	4±4	71± 5	29± 5
PMA	38±7	35±6	9± 7	91± 7
+amiloride	37±7	36±6	n.d.	n.d.
+staurosporine	4±2	n.d.	n.d.	n.d.
+PMA (24 h)	6±3	n.d.	0[a]	0[a]
4α-Phorbol 12,13-didecanoate	5±4	n.d.	n.d.	n.d.
Zymosan	38±7	40±10	44±14	57±14
Ionomycin	6±5	7±4	66± 2	34± 2
Arachidonic acid	4±4	2±4	66± 4	34± 4
Fluoride	6±3	5±5	45± 7	55± 7

(Table II). PMA and zymosan but not arachidonic acid or calcium ionophore have also been shown to be potent activators of PKC in liver macrophages (Table II, [10]). Therefore, it may be that the effect of fluoride on pH_i is also mediated by PKC. This assumption is confirmed by the observations that the fluoride-induced change in pH_i is suppressed by staurosporine or desensitization of PKC (data not shown).

In order to distinguish between the relative contributions of NADPH oxidase and PKC on pH_i and activity of Na^+/H^+ antiporter it would be necessary to perform experiments where (1) NADPH oxidase is activated by a PKC-independent pathway, and (2) when NADPH oxidase but not PKC activity is inhibited. However, all agents tested so far elicit superoxide release via activation of PKC ([9] and unpublished data) and reported inhibitors of NADPH oxidase [23,24] are inactive or cytotoxic in liver macrophages (unpublished data). Therefore, the relative contributions of NADPH oxidase and PKC in the zymosan- and PMA-elicited decrease of pH_i in liver macrophages remain open.

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