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Modulation of Na^+/H^+ exchange and intracellular pH by protein kinase C and protein phosphatase in blood platelets

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Phosphorylation of the Na^+/H^+ exchanger in human platelets is apparently controlled by the balancing activities of protein kinase C (PKC) and protein phosphatase (PP). To explore cellular expressions of these activities, we have examined the impact of modulation of PKC and PP on Na^+/H^+ exchange activity, its pH_i set point and intracellular pH (pH_i). These parameters were followed spectrofluorimetrically in BCECF-loaded platelets. Phorbol 12-myristate 13-acetate (PMA) and dihexanoylglycerol (DHG), which stimulate PKC, and okadaic acid, which inhibits PP 1 and 2A, elevate the measured parameters in concert, while staurosporine, which inhibits protein kinases, had opposite effects. The stimulatory and inhibitory effects are similarly very rapid, being discerned within seconds. It is concluded that: (a) phosphorylation of the Na^+/H^+ exchanger is the common origin of the diverse effects of PMA, DHG, okadaic acid and staurosporine, (b) Na^+/H^+ exchange properties are tightly regulated by phosphorylation and dephosphorylation, and (c) the exchanger plays a major role in pH_i regulation in platelets.

Introduction

The plasma membrane Na^+/H^+ exchanger is widespread and has been extensively studied (for review series: Refs. 1 and 2) but functional aspects remain to be clarified at the molecular level. It has been suggested that Na^+/H^+ exchange of blood platelets plays a role in several functions: activation of platelets by thrombin and other agonists [3–6], mobilization of arachidonic acid in response to 'weak agonists' (epinephrine, ADP and thrombin at low concentrations), dense granule secretion [7] and cytoplasmic pH regulation [6]. Na^+/H^+ exchange is also involved in regulatory volume decrease [8,9] and the exchange activity is correlated with the clinical manifestation of hypertension [10–12]. It is established that stimulation of platelets by thrombin and other inducers is accompanied by activation of Na^+/H^+ exchanger, but there is debate as to whether the activation is required for aggregation [13–16] and whether it is a prerequisite for Ca^{2+} mobilization [17–22].

It has been proposed that Na^+/H^+ exchange activity is regulated by several pathways, including allosteric activation by pH_i [23–26], covalent protein modification through protein kinase C (PKC) [27], phosphorylation-induced conformational change [27,28] and changes in the turnover or quantity of exchange units [29]. Platelet Na^+/H^+ exchanger appears to be tightly regulated by PKC [12].

The role of phosphorylation in the activation of the Na^+/H^+ exchange molecule in several cell types has recently been demonstrated [30]. A specific antibody toward the terminal cytoplasmic (carboxyl) end of the protein revealed that the exchanger is a 110 kDa glycoprotein that is phosphorylated in growing cells. Furthermore, activation of resting cells with either epidermal growth factor, thrombin, phorbol esters or serum stimulated phosphorylation of the Na^+/H^+ exchanger with a concomitant rise in intracellular pH.

We have examined the role of phosphorylation of the Na^+/H^+ exchanger in platelets by two converging approaches. In one approach [31], we have used the above-mentioned antibody and detected, for the first time, the exchanger in platelets and studied its phos-

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phorylation. Agents such as phorbol ester, diacylglycerol or okadaic acid, enhanced the phosphorylation of the exchanger, while staurosporine inhibited it [31]. In the second approach (present study), we have examined the impact of modulation of PKC and phosphatase on Na^+/H^+ exchange and on pH_i in platelets. These studies demonstrate that Na^+/H^+ exchange properties are tightly and markedly affected by phosphorylation and dephosphorylation and that the exchanger plays a major role in pH regulation in platelets.

Materials and Methods

Reagents

Albumin (bovine, fraction V), nigericin, *sn*-1,2-dihexanoylglycerol (DHG), amiloride, sodium propionate, probenecid and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. 2',7'-Biscarboxyethyl-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes. Staurosporine was from Calbiochem. 5-*N*-(3-Aminophenyl)amiloride was a gift from Dr. D. Cassel (Biology Department Technion-Israel Institute of Technology, Haifa). Okadaic acid was received from Fujisawa Pharmaceutical Co. The inhibitor R59022 was obtained from Janssen.

Solutions

The acid-citrate-dextrose solution was composed of 65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate. The standard Na^+ medium contained, in mM: 140 NaCl, 5 KCl, 0.42 NaH_2PO_4, 10 glucose and 20 Hepes (pH 7.35). For pH_i measurements for an extended period (Fig. 4), 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were added to the standard medium. The NaCl-Na propionate solution contained, in mM: 80 NaCl, 60 sodium propionate, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 Hepes (pH 7.35). The osmolarity of all media was adjusted to 285 mosM with distilled water or the major salt. Stock solution of nigericin (1 mM) and probenecid (0.1 M) were prepared in ethanol, while stock solutions of BCECF acetoxymethyl ester (2.5 mg/ml), PMA (1.6 mM), DHG (50 mM), R59022 (50 mM), and staurosporine (0.5 mM) were prepared in dimethylsulfoxide.

Preparation of platelet suspension

Venous blood was drawn from healthy volunteers, aged 25–55 years, who had not received any medication during the previous 10 days. The blood was anticoagulated with acid-citrate-dextrose at a volume ratio of blood: anticoagulant of 6:1. Platelet-rich plasma was obtained by centrifugation at $120 \times g$ for 10 min and had a pH of 6.5. For the spectrofluorimetric measurements of pH changes, the platelets were first loaded in the dark with the parent acetoxymethyl ester (3 μM, final) for 30 min at 23°C and were then gel-filtered through a Sepharose 2B column (10×0.76 cm). The solution used to equilibrate the column and to elute the platelets was the standard Na^+ medium described above but modified in that albumin (1 mg/ml) was added and the pH was adjusted to 6.8. The suspension of BCECF-loaded platelets was supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), hirudin (0.01 unit/ml) and probenecid (0.2 mM). Probenecid, (*p*-[dipropylsulfamoyl]benzoic acid), blocks organic anion transporters that remove fluorescent dyes from the cytoplasmic matrix [32]. We found that the use of probenecid (0.2 mM) cuts down the leak of BCECF from platelets about 3-fold, so that most of the fluorescence measurements were conducted at 4–6% leak (8.5% being the maximal level at the end of all measurements).

Determination of cytoplasmic pH changes

Changes in cytoplasmic pH were determined, essentially, as described [33,34] but with some modification. Fluorescence was measured in a Jasco FP-770 spectrofluorimeter with wavelength settings at 495 and 525 nm for excitation and emission, respectively, using 5 and 10 nm slits, respectively.

Na^+/H^+ exchange For measurement of Na^+/H^+ exchange an aliquot (10–20 μl) of the gel-filtered platelets loaded with BCECF, was mixed with 1.8 ml of the NaCl-Na propionate solution ($(2–3) \cdot 10^7$ platelets/ml). All tested compounds were added to the assay medium dissolved in 1.8 μl DMSO, prior to the addition of the platelets. The solvent was equally added to the control. Fluorimetric tracings were recorded for 90 s, starting within less than 3 s of the addition of the platelets. At termination, Triton X-100 (9 μl of 10%) was added and calibration of pH vs. fluorescence was performed with increments of 3-(*N*-morpholino)propanesulfonic acid as titrant. The pH was monitored in the cuvette by GK2401C combined electrode, connected to Ion 83 Ion meter (Radiometer, Copenhagen), with a resolution of 0.001 pH unit. A predetermined factor used to correct for the red shift of the intracellular dye and the calibration of pH_i were as described [33–35]. The Na^+/H^+ exchange rate was determined by ΔpH_i per 9 s at pH_i 7.0. The set point is the pH_i in which the exchange rate is nil, an extremely low rate, chosen arbitrarily but uniformly throughout. It was calculated by determining the rates of the Na^+/H^+ exchange at several pH_i values (at least seven measurements) and extrapolating the curve (log rate vs. pH_i, with regression coefficients > 0.94) to a pH_i for which the rate is 0.001 pH/9 s (10-fold lower than the smallest discernible rate).

pH_i changes. For measurements of pH_i changes the platelets were suspended in the standard NaCl medium, pH 7.35, containing the compound being tested and assayed fluorimetrically as above but for extended periods. The pH_i of untreated gel-filtered platelets was

7.30 ± 0.05 ($n = 15$). All fluorimetric measurements were conducted within 60 min after the preparation of dye-loaded platelets, with a maximal dye leak of $8.5 \pm 0.7\%$ ($n = 21$).

Results

Effect of PKC and phosphatase modulation on Na^+/H^+ exchange

When platelets are suspended in isotonic medium, pH 7.35, containing NaCl and 60 mM sodium propionate, a rapid intracellular acidification occurs (≤ 3 s, within the time resolution of the mixing and the recording), to pH 6.83 ± 0.02 ($n = 36$). Thereafter gradual alkalinization becomes apparent. The alkalinization represents Na^+/H^+ exchange, since it is dependent on external Na^+ and is inhibited by over 90% by amiloride (200 μM) or 5-*N*-(3-aminophenyl)amiloride (20 μM). If the NaCl-Na propionate medium contains either PMA (0.2 μM) or okadaic acid (1 μM), the suspended platelets respond by increased rates of Na^+/H^+ exchange (Fig. 1). While the tracing of the PMA treatment is entirely distinct, the okadaic acid and control tracings display similar slopes after an initial period of about 25 s.

Table I compares the effects of PMA, okadaic acid, or both, on Na^+/H^+ exchange rate and set point. PMA significantly affects both parameters: it promotes the exchange rate and causes an alkaline shift in the pH_i set point. Okadaic acid promotes the exchange rate, significantly, but does not modify the set point. The combined effect of PMA and okadaic acid on the exchange rate is less than additive, suggesting a common site of action.

PMA is known to modulate PKC [36–38]. We also examined the effect of diacyl glycerol, a specific activator of PKC. Exogenous *sn*-1,2-dihexanoylglycerol (DHG) penetrates platelets readily and functions as a bioregulator of PKC [39]. Similarly to PMA, DHG stimulates Na^+/H^+ exchange rapidly. Fig. 2 shows

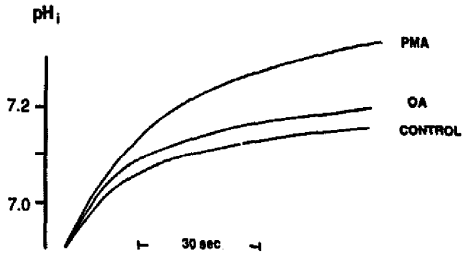

Fig. 1. Rate of alkalinization of platelets suspended in isotonic medium (pH 7.35), containing 60 mM sodium propionate, as affected by 0.2 μM phorbol 12-myristate 13-acetate (PMA) or 1 μM okadaic acid (OA). Shown are tracings of a single experiment representative of at least five additional experiments.

TABLE I

Platelet Na^+/H^+ exchange rate and set point: differential effects of PMA and okadaic acid

Given are average values ± S.E. of three experiments, each in triplicate. The rate is at pH_i 7.0. DMSO (1.8 μl), used as a solvent, was added to the control platelets as well. For rate: b vs. d, $P < 0.01$, all other comparisons, $P < 0.001$; for set point: a vs. c and b vs. d, no significant difference, all other comparisons, $P < 0.001$.

Treatment	Rate, ΔpH_i/9 s	Set point, pH_i
a Control	0.043 ± 0.001	7.32 ± 0.01
b PMA, 0.2 μM	0.092 ± 0.006	7.58 ± 0.01
c Okadaic acid, 1 μM	0.056 ± 0.002	7.33 ± 0.01
d PMA + okadaic acid	0.114 ± 0.002	7.56 ± 0.05

that the stimulation by DHG is concentration-dependent, exhibiting a saturation pattern, with $K_{0.5} = 5.1 \pm 1.1$ μM ($n = 4$). Fig. 2 further shows that, coupled with the stimulated rate, DHG causes an alkaline shift of the exchange pH_i set point, with a similar concentration dependence: $K_{0.5} = 4.4 \pm 0.5$ μM ($n = 4$). In contrast, staurosporine, a potent kinase inhibitor, results in a reduced Na^+/H^+ exchange rate with $K_I = 18.3 \pm 2.2$ nM ($n = 6$), coupled with a concordant acidic shift of the pH_i set point (Fig. 3).

Effect of PKC and phosphatase modulation on pH_i

In unstimulated non-epithelial cell types the Na^+/H^+ exchanger appears to be nearly quiescent when the cytoplasmic pH is in the physiological range but is increasingly active as pH_i is experimentally reduced below the set point [40]. Blood platelets exhibit this property as well [33]. It has been shown above that the rate and set point of Na^+/H^+ exchange are affected by modulation of the phosphorylative state of the exchanger. Therefore, this modulation may also be expected to affect the steady state pH_i if the exchanger

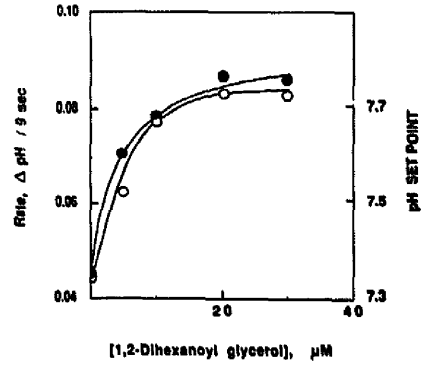

Fig. 2. Effect of 1,2-dihexanoyl glycerol concentration on Na^+/H^+ exchange rate (○) and pH_i set point (●). The exchange rate is measured at pH_i 7.0. Representative of four experiments.

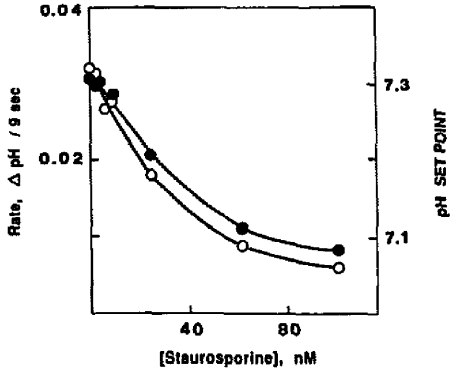

Fig. 3. Effect of staurosporine concentration on Na^+/H^+ exchange rate (○) and pH_i set point (●) in human platelets. The rate is measured at pH_i 7.0. Representative of six experiments.

plays a major role in the regulation of pH_i. Indeed, Fig. 4 shows that 0.2 μM PMA causes an upward shift in pH_i ($+0.14 \pm 0.01$ units, $n = 6$) while 0.1 μM staurosporine produces an opposite effect, (-0.20 ± 0.03 units, $n = 5$). Half maximal concentration for the effect of staurosporine is 24 nM. These changes persist for at least 30 min (the duration of the measurements).

DHG (25 μM) causes an increase of pH (about 0.1 unit) which peaks within less than 1 min but, thereafter, it gradually regresses, possibly due to its conversion by diacylglycerol kinase. Indeed, when an inhibitor of diacylglycerol kinase (R59022, 25 μM) is added together with DHG, the elevated pH is sustained for at least 30 min, in harmony with the effect of R59022 in

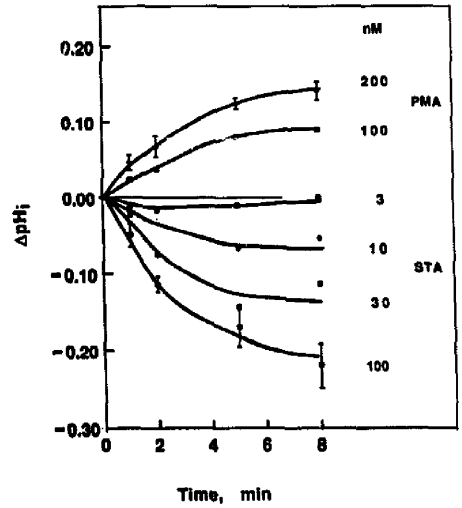

Fig. 4. Effects of phorbol 12-myristate 13-acetate (PMA) and staurosporine (STA) on intracellular pH of human platelets. The bars represent the standard error for five or six experiments; otherwise, each point is the mean of two experiments. The initial pH_i (time 0) was 7.33 ± 0.02.

the intact platelet [41,42]. The kinase inhibitor itself causes pH rise of about 0.05. Similarly, okadaic acid (1 μM) causes an alkaline shift of pH_i (0.05 ± 0.01, $n = 6$), but this results from a different mechanism, namely, inhibition of protein phosphatase [43].

The effects of staurosporine, PMA and dihexanoylglycerol does not result from an indirect effect on BCECF fluorescence, due to possible shape change [44], since no shape change can be observed turbidometrically, using a sensitive aggregometer. In contrast, under the same conditions, thrombin (0.1 U/ml) clearly affects the platelets' shape.

Discussion

Activation of the Na^+/H^+ exchanger and a resulting cellular alkalinization is a general response to agonists which activate the protein kinase C signalling pathway [25,45,46]. The present study shows that it holds true for human blood platelets as well. Moreover, the study further characterizes the regulation of Na^+/H^+ exchange and pH_i in platelets.

Several lines of evidence indicate that PKC is the kinase that predominantly regulates Na^+/H^+ exchange in platelets and thus plays a major role in pH_i control. (a) PMA affects, in concert, four parameters: it increases the exchange rate, it causes an alkaline shift of the pH_i set point, it leads to cellular alkalinization (this study) and it enhances the phosphorylation of the Na^+/H^+ exchanger [31]. All these effects are apparent concomitantly at the same PMA concentration. (b) Similarly, DHG affects all the four parameters in concert as well. The $K_{0.5}$ values for the action of DHG in elevating the Na^+/H^+ rate (5.1 ± 1.1 μM) and set point (4.4 ± 0.5 μM) are very close to its $K_{0.5}$ value when stimulating the phosphorylation of pleckstrin in intact platelets (3.4 ± 1.8 μM, in preparation). This observation is significant, since pleckstrin is a specific substrate for PKC [47,48] and DHG is known to regulate PKC in intact platelets [39]. (c) Staurosporine markedly affects the same parameters, but in an opposite direction: it inhibits the Na^+/H^+ exchange rate ($K_I = 16.3$ nM), it shifts the set point to a more acidic pH_i and it lowers the intracellular pH ($K_I = 24$ nM); these are presently shown. In addition, it curtails the phosphorylation of the transporter, induced by PMA or DHG [31]. The high potency of staurosporine in these four expressions is notable. Furthermore, these K_I values are even lower than the K_I (approx. 70 nM) observed for the inhibition of phosphorylation of pleckstrin by PKC in intact platelets [49]. Staurosporine is known to be a potent kinase inhibitor, but its specificity is under debate. At 0°C and after 1 h incubation, it binds equally to several purified kinases (protein kinase C, cAMP-dependent protein kinase, tyrosine protein kinase and calcium/calmodu-

lin-dependent protein kinase), indicating a non-specific interaction [50]. Yet, staurosporine is selectively more inhibitory toward PKC than to other kinases at the nanomolar range [51].

The control of platelet Na^+/H^+ exchange by phosphorylation exhibits some intriguing properties. (a) The control is dynamic: PMA, DHG and staurosporine elicit their responses, indicated above, very rapidly, i.e. within 3 s. This interval includes the time required for the reagents to penetrate the platelets. (b) The control is tight: at saturation (≈ 25 μM for DHG, ≈ 200 nM for PMA), these reagents stimulate the exchange rate by more than 2-fold, but only approx. 10% of the stimulated rate is observed in the presence of staurosporine. (c) The control involves protein phosphatase, which counteracts the kinase activity. The involvement of phosphatase is demonstrated by two findings: the inhibitory effects of staurosporine, allowing the expression of the phosphatase activity, and the stimulatory effects of okadaic acid. Okadaic acid stimulates the exchange rate but, unlike PMA, it does not appear to change the set point (Fig 1, Table I). It may reflect the facts that okadaic acid is less potent than PMA and that the exhange rate is a more sensitive measure for stimulating effects than the set point. Furthermore, in addition to phosphatases 1 and 2A, that are inhibited by okadaic acid at 1 μM [43], other phosphatases may be involved as well. The kinase and phosphatase, by their counteracting effects, maintain a fine balance of the regulation of the Na^+/H^+ exchanger by covalent bonds. Whether the C kinase and phosphatase affect the transporter directly, or through another 'integrator', as demonstrated, for example for mitogen-activated protein kinase [52], remains to be elucidated. Furthermore, we have so far considered intracellular pH as being related merely to Na^+/H^+ exchange, but clearly, other factors could be involved [53–57].

The presently reported mean pH_i value of the BCECF-loaded platelets (7.30) is similar to previous reports in which the same procedure for platelet preparation has been used [14,16,33,58]. This value is higher than those reported elsewhere, e.g. pH 7.09 [6] or 7.15 ± 0.05 [22], when the platelets were prepared in another way. A study in progress in our laboratory indicates that the labelling step indeed affects the pH_i value, at least partly due to an effect on Na^+/H^+ exchange. Until the exact pH_i is determined, the pattern of pH_i changes are considered, rather than their absolute values.

Our experiments were performed in nominally bicarbonate free media. In aortic smooth muscle cells bicarbonate is required for the activation of Na^+/H^+ exchanger by phorbol esters [56]. However, concerning blood platelets, it has been reported recently that bicarbonate does not modify the pattern of agonist response [22], and that the net extrusion of H^+ from stimulated platelets is predominantly mediated by Na^+/H^+ exchange without an apparent contribution of HCO_3^-/Cl^- exchange [59].

The present study supports an earlier report by Ozaki et al. [60]. They have used dioctanoylglycerol to activate PKC in platelets and concluded that PKC has a dual role in pH_i regulation, adjusting the pH_i value at a certain set point on the one hand, and increasing the rate constant of the Na^+/H^+ exhanges on the other.

The balancing effects of a kinase and phosphatase in determining the phosphorylative state of proteins, play major roles in regulations in many cell types. Furthermore, an array of kinases and phosphatases may participate in these control mechanisms. These principles are illustrated by our study, which deals with just one cell type and with protein kinase C as well as phosphatase 1 or 2A.

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