Arachidonic acid activates a proton conductance pathway and the Na^+/H^+ exchanger in platelets

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The treatment of aspirinated platelets with the endomembrane Ca^{2+} -ATPase inhibitor thapsigargin (Tg) induces a large increase in cytosolic pH (pH_i), as measured with the intracellular fluorescent indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein. In contrast, Tg induces a decrease in pH_i in the presence of the Na^+/H^+ exchanger inhibitor 5-(*N,N*-hexamethylene)-amiloride (NHA). Both effects are inhibited if the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is chelated by loading with bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester (BAPTA-AM). Without BAPTA, the pH effects are inhibited in the presence of BSA or the phospholipase A_2 inhibitor oleoyloxyethylphosphocholine. These observations are consistent with the Tg-induced pH effects being mediated at least in part by the release of arachidonic acid (ArA) on activation of phospholipase A_2 by the increased $[\text{Ca}^{2+}]_i$. Exogenous ArA promotes a rapid decrease in pH_i in platelets suspended in a high- $[\text{Na}^+]$ medium, and an increase in pH_i if platelets are depolarized by suspension in a high- $[\text{K}^+]$ medium in

the presence of valinomycin and the external pH is increased to 7.9. The protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) behaves like ArA, although ArA is not a protonophore. It is concluded that ArA activates a proton conductance across the plasma membrane. The latter is inhibited by La^{3+} . In high- $[\text{Na}^+]$ media, the pH_i previously decreased by ArA recovers rapidly on removal of ArA with BSA. The effect is prevented by NHA. The recovery after BSA is much slower if FCCP rather than ArA is used to decrease pH_i , but it is fast again with both ArA and FCCP. Furthermore, pH_i previously decreased by ArA also recovers readily on inhibition of the ArA-activated H^+ conductance with La^{3+} , and the effect is NHA-sensitive. When pH_i is decreased with the K^+/H^+ ionophore nigericin, a rapid recovery is activated by ArA followed by BSA (but not by BSA alone). The effect is independent of Ca^{2+} and protein kinase C. It is concluded that ArA, besides activating the H^+ conductance, also acts as an activator of the Na^+/H^+ exchanger.

INTRODUCTION

There is a general consensus that the thrombin-induced alterations in cytosolic pH (pH_i) in human platelets comprise two phases: an initial transient acidification followed by a phase of prolonged alkalinization [1–5]. The initial phase of platelet acidification was proposed to be an artifact, reflecting platelet shape change [5], but this concept has been contested [3]. The second phase seems to reflect the activation of the Na^+/H^+ exchanger, as it is not observed in the presence of inhibitory amiloride analogues, or in Na^+ -free media [1–6]. The activation of the Na^+/H^+ exchanger seems to be mediated by the stimulation of protein kinase C (PKC) [1,5,8–12] as well as by the rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [6,7]. When the antiporter is not operative, the agonist-induced intraplatelet alkalinization is replaced by a sharp decrease in pH_i , of which the origin is not totally clear. It has been ascribed to an increased production of H^+ because of increased metabolic demands [1,13] or to the stimulation of the Ca^{2+} -ATPase associated with the plasma membrane, which in erythrocytes exchanges external H^+ for internal Ca^{2+} [14,15]. A Ca^{2+} -ATPase is present in the platelet plasma membrane [16,17] and is stimulated by the activation of PKC [18,19].

The specific inhibitor of the endomembrane Ca^{2+} -ATPase, thapsigargin (Tg), elevates cytosolic Ca^{2+} in an agonist-independent fashion, and in turn the partial depletion of the Ca^{2+}

stores activates a pathway of Ca^{2+} influx from the extracellular environment [20–23]. Also Tg, similarly to thrombin, influences pH_i in platelets by promoting alkalinization, or alternatively acidification if the Na^+/H^+ exchanger is not operative [24].

This study analyses in greater detail the properties of the Tg-induced changes in pH_i and identifies arachidonic acid (ArA) as one mediator of the Tg effects. It also reports on the effects of exogenous ArA on platelet pH_i .

MATERIALS AND METHODS

Materials

Hirudin, apyrase, ionomycin, thapsigargin, oleoyloxyethylphosphocholine (OOPC), 5-(*N,N*-hexamethylene)-amiloride (NHA), ethylisopropyl amiloride, ArA (diluted to 15 mM in ethanol under N_2 , divided into small aliquots and maintained at -70°C until use), BSA (fraction V, essentially fatty acid-free), bis-indolylmaleimide (GF 109203X) and staurosporine were purchased from Sigma (St. Louis, MO, U.S.A.); 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester (BCECF-AM), bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester (BAPTA-AM) and 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetra-acetic acid penta-acetoxymethyl ester (INDO 1-AM) were from Calbiochem; prostacyclin (prostaglandin I_2 ; PGI_2) and the trifluoromethyl ketone analogue

Abbreviations used: ArA, arachidonic acid; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra-acetoxymethyl ester; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester; GF 109203X, bis-indolylmaleimide; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; INDO 1-AM, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N,N,N',N'*-tetra-acetic acid penta-acetoxymethyl ester; NHA, 5-(*N,N*-hexamethylene)-amiloride; NMG, *N*-methylglucamine; OOPC, oleoyloxyethylphosphocholine; PGI_2 , prostacyclin (prostaglandin I_2); pH_i , cytosolic pH; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Tg, thapsigargin.

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of ArA were from Cascade Biochem Ltd.; [5,6,8,9,11,12,14,15-³H]ArA was from Amersham; BW 755C was from Wellcome Ltd.; and dithiocarbocyanine was from Molecular Probes. All other reagents were of analytical grade.

Platelet preparation

Platelet-rich plasma and washed platelets were prepared and treated with aspirin as previously reported [25] from fresh blood drawn from healthy volunteers and mixed with acid citrate/dextrose anticoagulant supplemented with hirudin (50 m-units/ml), apyrase (80 m-units/ml) and PGI₂ (0.2 µg/ml). Platelets were kept at room temperature until use.

Determination of cytosolic pH

The intracellular pH was determined with the fluorescent pH indicator BCECF. The pH-sensitive fluorescent probe BCECF-AM (5 µM) was added to aspirinated platelet-rich plasma and loading was performed for 30 min at 25 °C. Unless otherwise stated, the pellet was resuspended in Tyrode buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM Na/Hepes, 10 mM glucose, pH 7.4) in the presence of 40 m-units/ml apyrase and 5 m-units/ml hirudin; 0.2 mM EGTA was added at the beginning of each incubation. The platelets were used at a concentration of (0.5–1) × 10⁸/ml. Fluorescence was measured at 25 °C in a thermostatically controlled, magnetically stirred cuvette in a Shimadzu RL-5000 spectrofluorimeter with excitation and emission wavelengths set at 503 and 530 nm respectively. Most experiments were performed under these temperature conditions because this decreased the equilibration time and the changes in BCECF fluorescence with changing temperature. Critical experiments were also performed at 37 °C, with superimposable results. Titration of the pH range was performed with a pH electrode at the end of the tracings after permeabilization with saponin (200 µg/ml) by addition of calibrated aliquots of NaOH or HCl. This method allows calibrations at the end of each trace under all incubation conditions. The results are essentially identical with those obtained with the nigericin titrations in KCl-based media [1]. The interference of BSA, mainly attributable to the contaminating external BCECF, was minimal in intact platelets and was subtracted from the relevant tracings. Similar subtractions were performed for other interfering substances.

Chelation of internal Ca²⁺

This was obtained by addition to platelet-rich plasma of 50 µM BAPTA-AM 20 min after BCECF-AM, and prolonging the incubation for a further 20–30 min.

Measurement of PLA₂ activity

PLA₂ activity was measured as [³H]ArA release. Washed aspirinated platelets resuspended in Tyrode medium (buffered at pH 6.4 with citric acid) at a concentration of 3 × 10⁹/ml were loaded with 3 µCi of carrier-free [³H]ArA for 90 min at room temperature in the presence of hirudin (50 m-units/ml), apyrase (80 m-units/ml) and PGI₂ (0.2 µg/ml). After dilution 5:1 in the same medium containing 3 mg/ml of BSA, and centrifugation at 700 g for 12 min at room temperature, the pellet was washed again and the cells were resuspended in Tyrode buffer at pH 7.4 at a concentration of 5 × 10⁸/ml. Platelets were incubated at 25 °C in Na-Tyrode buffer supplemented with Ca²⁺ (final concentration 0.8 mM). OOPC (30 µM) was preincubated for 3 min before the addition of Tg (zero time; see inset to Figure 2). At the indicated times aliquots were withdrawn and the reaction was stopped by dilution with the same volume of an ice-cold mixture

of 75 mM sodium-phosphate buffer, pH 7.4, containing 4 mM EDTA and 6% (v/v) glutardialdehyde. After centrifugation at 10000 g for 3 min at room temperature, the [³H]ArA released was measured in the supernatant by liquid-scintillation counting. Values of [³H]ArA release were expressed as a percentage of the total radioactive counts of the corresponding suspension solubilized with 1% (v/v) Triton X-100.

Measurement of [Ca²⁺]_i

This was performed in INDO 1-AM-loaded platelets as reported in [23] with excitation and emission wavelengths set at 340 and 400 nm respectively.

Membrane potential

This was measured by following the quenching of fluorescence of dithiocarbocyanine on its distribution on the plasma membrane as indicated in [26] at 620 and 660 nm excitation and emission wavelengths respectively.

RESULTS

The treatment of aspirinated platelets with the endomembrane Ca²⁺-pump inhibitor Tg promotes the partial release of Ca²⁺ from the intracellular deposits, leading to an increase in [Ca²⁺]_i. In turn the depletion of the stores activates an influx of Ca²⁺ from the medium if the experiment is conducted in the presence of Ca²⁺. As shown in Figure 1(A) (see also [24]), the addition of Tg is also followed by a large increase in pH_i. Such an alkalization is replaced by a net acidification if the experiment is conducted in the presence of the Na⁺/H⁺ exchanger inhibitors NHA (Figure 1B) or ethylisopropyl amiloride (owing to the slightly higher inhibitory efficacy of NHA, this amiloride derivative was predominantly used in this study). Tg also promotes

Figure 1 Ca²⁺ dependence and BSA sensitivity of the Tg-induced variations in pH_i in the absence and presence of NHA

(A–E) The fluorescence variations induced by 400 nM Tg in platelets preloaded with 5 µM BCECF-AM and resuspended in Na⁺-Tyrode (A–D) or in a medium in which NaCl was replaced by 145 mM NMG (E) as described in the Materials and methods section. Where indicated the external free Ca²⁺ concentration was 0.8 mM, that of BSA was 0.2 mg/ml and that of NHA was 20 µM. The broken lines marked BAPTA indicate the Tg-induced variations of pH_i in platelets co-loaded with BCECF-AM and BAPTA-AM (see the Materials and methods section) and incubated in the absence of added Ca²⁺. (F) The increase in [Ca²⁺]_i induced by Tg in the presence of 0.8 mM external Ca²⁺ in INDO 1-AM-loaded platelets. Solid line, no BSA; dotted line, 0.2 mg/ml BSA. The traces are representative of duplicate experiments with at least five different preparations with similar results.

Figure 2 The Tg-induced variations of pH_i are sensitive to the inhibition of phospholipase A_2

BCECF-loaded platelets were incubated in Na^+ -Tyrode. The effect of OOPC (20 μ M, broken lines) on the Tg-induced variations in pH_i are shown in the presence (A) and absence (B) of NHA. The traces are representative of duplicate experiments with at least five different preparations with similar results. Inset: the Tg-induced release of [3H]ArA in the absence and presence of OOPC. The concentration of free Ca^{2+} was 0.8 mM; Tg was added at zero time. When present, OOPC (30 μ M) was added 3 min before Tg. The points are taken from duplicate determinations from two platelet preparations. See the Materials and methods section for details.

the decrease of pH_i in the absence of NHA if the experiment is performed in a Na^+ -free medium where *N*-methylglucamine (NMG) substitutes for Na^+ (Figure 1E). These experiments show that Tg promotes the activation of the Na^+/H^+ exchanger and, at the same time, also of some acid-producing process. The latter is masked by the operation of the Na^+/H^+ exchanger when its activity is allowed to proceed unchecked.

The alkalization by Tg is maximal in the presence of extracellular Ca^{2+} , and it is almost completely abolished if platelets are loaded with the plasma membrane-permeable Ca^{2+} chelator BAPTA-AM. In addition the acidification observed in the presence of NHA is highest with external Ca^{2+} and very low in BAPTA-AM-loaded platelets (Figures 1A and B). These results establish the Ca^{2+} -dependence of the Tg-promoted effects.

The Tg-induced alkalization decreases if BSA is included in the incubation medium (Figure 1C). This observation suggests that the operation of the Na^+/H^+ exchanger is stimulated by some agent removed by BSA. The same or a similar agent also seems to activate the acid-producing activity observed in the presence of NHA. In fact, BSA decreases the Tg-induced decrease in pH_i observed when the Na^+/H^+ exchanger is inhibited (Figure 1D). Control experiments performed in INDO 1-loaded platelets show that BSA does not interfere with the increase in [Ca^{2+}] $_i$ induced by Tg (Figure 1F).

We reasoned that the agent removed by BSA could be ArA produced on activation of phospholipase A_2 by the increased [Ca^{2+}] $_i$. ArA is indeed produced on treatment of platelets with Tg, and its production is depressed by the phospholipase A_2 inhibitor OOPC (Figure 2, inset). Thus OOPC decreases the Tg-promoted increase of pH_i (Figure 2B) as well as the Tg-induced acidification monitored when the Na^+/H^+ exchanger is inhibited (Figure 2A). Similar results were obtained with mepacrine; however, it was a less powerful inhibitor of the phospholipase. Other inhibitors, such as bromophenacylbromide and the trifluoromethyl ketone analogue of ArA, had no effect on the Tg-induced ArA production. A control further supporting the hypothesis that ArA is indeed the BSA-removable species re-

Figure 3 ArA-treated BSA is less effective than defatted BSA in inhibiting the Tg-induced decrease of pH_i in the presence of NHA

BCECF-loaded platelets were incubated in Na^+ -Tyrode. When indicated, the concentration of BSA (fatty acid-free, broken line) or premixed with ArA (1:4 molar ratio of BSA to ArA, dotted line) was 0.5 mg/ml. The traces are representative of duplicate experiments with two different preparations with similar results.

Figure 4 ArA and FCCP promote the decrease of pH_i in platelets resuspended in low- K^+ buffers

BCECF-loaded platelets were resuspended in Na^+ -Tyrode (A, B) or in a medium in which NaCl was replaced by 145 mM NMG (C). ArA (solid line) was present at 8 μ M and FCCP (broken line) at 0.12 μ M. The traces are representative of duplicate experiments with at least three different preparations with similar results.

sponsible for the effect of BSA on the Tg-induced changes in pH_i is reported in Figure 3. It is shown that ArA-treated BSA (approx. 4:1 molar ratio) is less effective than fatty acid-free BSA in counteracting the decrease in pH_i induced by Tg in NHA-treated platelets.

The above observations suggest that ArA, produced on increasing [Ca^{2+}] $_i$, might be involved in the control of the Na^+/H^+ exchanger as well as in the acidification monitored when the exchanger is not operative. We therefore performed experiments to study the effect of exogenous ArA on platelet pH_i .

ArA opens a proton conductance pathway in the platelet plasma membrane

As shown in Figure 4(A), the addition of 8 μ M ArA to platelets suspended in a Na^+ -rich medium evokes a rapid decrease in pH_i .

Figure 5 ArA and FCCP promoted decrease of pH_i in Na^+ -Tyrode is enhanced by valinomycin

BCECF-loaded platelets were resuspended in Na^+ -Tyrode. Valinomycin (Valino) was present at $0.33 \mu\text{g/ml}$, ArA at $10 \mu\text{M}$ and FCCP at $0.2 \mu\text{M}$. The traces are representative of duplicate experiments with three different preparations.

Figure 6 Membrane potential variations induced by ArA, FCCP, NHA and valinomycin

Platelets were resuspended in Na^+ -Tyrode and treated with $0.6 \mu\text{M}$ diethiocarbocyanine (whose accumulation in the polarized plasma membrane induces a decrease of the fluorescence). ArA was present at $10 \mu\text{M}$, FCCP at $0.2 \mu\text{M}$, NHA at $20 \mu\text{M}$, gramicidin (Gram) at $1 \mu\text{M}$ and valinomycin (Valino) at $0.33 \mu\text{g/ml}$. (C) The depolarizing effect of sequential pulses of 10 mM KCl in valinomycin-hyperpolarized platelets.

The effect of ArA is mimicked by the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore that allows the distribution of H^+ according to its electrochemical potential across the plasma membrane. With both agents, the acidification was more extensive if the experiment was performed with NHA (Figure 4B), or in a medium where Na^+ was replaced by NMG (Figure 4C). Under the conditions of Figures 4(A) and 4(B), the increase of the ArA-induced decrease in pH_i in the presence of NHA was 40 ± 10 (S.D.)% ($n = 10$, $P < 0.001$). These experiments show that the decrease in pH_i promoted by ArA (and to a certain extent also by FCCP) is antagonized by the activity of the Na^+/H^+ exchanger.

The similarity between ArA and FCCP might indicate that ArA opens a proton-permeable path across the platelet plasma membrane. Thus if the experiments are conducted in the presence of the K^+ ionophore valinomycin, which hyperpolarizes the plasma membrane in low- K^+ media, the decrease in pH_i after ArA (or FCCP) is larger (Figure 5). Both ArA and FCCP, by opening a proton conductance pathway, bring about a large decrease in the plasma membrane potential. A further depolar-

Figure 7 ArA and FCCP promote the increase in pH_i in platelets resuspended in a high- K^+ medium, at high external pH ; effect of La^{3+}

BCECF-loaded platelets were resuspended in a medium composed of 135 mM KCl, 15 mM NaCl, 1 mM $MgCl_2$, 20 mM Na/Hepes, 10 mM glucose, pH 7.4, in the presence of $20 \mu\text{M}$ NHA. Where indicated, the external pH was increased to 7.9 with Tris base (10 mM). Valinomycin (Valino) was present at $0.33 \mu\text{g/ml}$, ArA at $8 \mu\text{M}$ and FCCP at $0.25 \mu\text{M}$. The $LaCl_3$ concentration (broken line) was $20 \mu\text{M}$. The traces are representative of duplicate experiments with at least four different preparations with similar results.

ization is induced by NHA. As expected, valinomycin promotes a hyperpolarization (Figure 6). To test further the hypothesis that ArA opens a proton conductance pathway across the plasma membrane, the platelets were resuspended in a high- K^+ medium and supplemented with valinomycin (or with the K^+ and Na^+ ionophore gramicidin; results not shown). This treatment depolarizes the plasma membrane and renders it permeable to potassium ions. Furthermore, the external pH was increased to 7.9 with Tris base (the rapid fluorescence increase immediately after the addition of Tris is largely due to extracellular BCECF). In these conditions the net electrochemical H^+ gradient is directed outward and the opening of a proton conductance pathway is expected to increase pH_i . Thus an increase in pH_i is promoted by FCCP (Figure 7A). A similar alkalinization is also induced by ArA (Figure 7B) after an initial transient decrease of pH_i , probably due to the permeation of the neutral undissociated acid across the plasma membrane and its dissociation within the cytosol. These experiments establish that ArA activates a proton conductance pathway across the plasma membrane of platelets, leading to the distribution of H^+ according to its electrochemical gradient. The effect of ArA is similar to that of the classic protonophore FCCP, although ArA is not a protonophore. The platelet plasma membrane is impermeable to H^+ before the addition of ArA or FCCP, and also when the conductance to K^+ (and Na^+) are increased by valinomycin (or gramicidin).

The ArA-promoted increase in the H^+ conductance is almost completely inhibited by $20 \mu\text{M}$ La^{3+} . The effect of La^{3+} is specific for ArA, as it does not interfere with the alkalinization induced by FCCP. The effect of La^{3+} seems not to be due to the formation of insoluble salts with ArA, because no turbidity was observed several hours after mixing ArA and La^{3+} in the same incubation medium (without platelets). Some turbidity appeared with the same ArA concentration but with 8–10-fold higher La^{3+} concentrations. The activation of the H^+ conductance by ArA is unaffected by the protein kinase inhibitor staurosporine, by the PKC inhibitor GF 109203X or by loading the platelets with the Ca^{2+} chelator BAPTA-AM.

Figure 8 The recovery of pH_i decreased by ArA or FCCP on their removal with BSA

BCECF-loaded platelets were resuspended in Na⁺-Tyrode. When indicated, ArA was present at 8 μ M, FCCP at 0.15 μ g/ml and NHA at 20 μ M (broken line). The BSA concentration was 0.2 or 1.2 mg/ml. The traces are representative of duplicate experiments with at least three different preparations with similar results.

ArA activates the Na⁺/H⁺ exchanger

The decrease in pH_i induced by ArA in platelets suspended in a high-Na⁺ medium is readily reversed if the fatty acid is removed with BSA (Figure 8A). The rapid recovery of pH_i frequently leads to a temporary hyperalkalinization. In control experiments, this amount of BSA (0.2 mg/ml) was shown to fully prevent the decrease of pH_i by subsequently added ArA (Figure 8A). As described above, the ArA-induced acidification is potentiated by NHA. Under these conditions the increase in pH_i induced by the removal of ArA with BSA was almost completely abolished (after an initial rapid phase, probably representing the efflux of undissociated ArA). These experiments show that the increase of pH_i on removal of the fatty acid depends on the activity of the Na⁺/H⁺ exchanger.

As with ArA, FCCP can also be removed by sufficiently high concentrations of BSA. As shown in Figure 8(B), the FCCP-induced decrease of pH_i is followed by a rather slow alkalinization on removal of the protonophore. This is at variance with what is observed with ArA. It seems that a similar decrease in pH_i to that induced by ArA or by the uncoupler does not activate equally the Na⁺/H⁺ exchanger and that the fatty acid itself acts as an activator. Thus if platelets are first treated with FCCP and then with ArA, the addition of the latter promotes a further decrease in pH_i, due in part to the influx of the undissociated fatty acid, followed by a spontaneous partial alkalinization; the subsequent addition of BSA rapidly completes the recovery of pH_i (Figure 8C). Thus the ArA-induced activation of the Na⁺/H⁺ antiporter is also visible in the presence of FCCP. The fatty acid that interacts with the antiporter seems not to be readily removed by BSA, because its activating effect is observed after adding BSA, which binds ArA (and FCCP) strongly. Alternatively the activation might be operated by some derivative of the fatty acid, whose production does not require the operation of the cyclooxygenase or the lipoxygenase, however, being unaffected by the lipoxygenase inhibitor BW 755C. Identical results are obtained with non-aspirinated platelets (results not shown).

The ArA-promoted activation of the exchanger is also observable if the ArA-activated proton conductance is decreased with La³⁺ rather than by removal of the fatty acid with BSA. In fact La³⁺, which strongly depresses the cytosolic acidification otherwise promoted by the subsequent addition of ArA (Figure

Figure 9 The La³⁺ inhibition of the ArA-induced H⁺ permeability reveals the ArA-dependent stimulation of the Na⁺/H⁺ exchanger

BCECF-loaded platelets were resuspended in Na⁺-Tyrode but EGTA was omitted. Where indicated, the LaCl₃ concentration was 20 μ M. The trace with NHA is represented by the broken line. The traces are representative of duplicate experiments with at least three different preparations with similar results.

Figure 10 Effect of Tg and PMA on the ArA-induced decrease of pH_i

BCECF-loaded platelets were resuspended in Na⁺-Tyrode. Free Ca²⁺ was present at 0.8 mM and ArA at 5 μ M. (A) Tg (400 nM) or PMA (50 nM) was added at the arrow, in the absence (solid line) or in the presence (broken line) of NHA. (B) Tg (400 nM) followed by BSA (0.2 mg/ml) (broken line) or BSA alone (solid line) was added as indicated. The traces are representative of duplicate experiments with at least two different preparations with similar results.

9B), induces a rapid amiloride-sensitive recovery of pH_i if it is added after ArA (Figure 9A).

Figure 10 shows that a partial recovery of pH_i, previously decreased by ArA, is induced by Tg, which increases [Ca²⁺]_i, as well as by phorbol 12-myristate 13-acetate (PMA), which activates PKC. Both effects are abolished by NHA. These results are in accordance with reports showing that the increase in [Ca²⁺]_i or the activation of PKC act as activators of the Na⁺/H⁺ exchanger. As might be expected, the recovery of pH_i on addition of BSA is more extensive and its rate is faster if BSA is preceded by Tg (Figure 10B).

Figure 11 The effect of Tg, PMA and ArA on nigericin-acidified platelet cytosol

(A) BCECF-loaded platelets were resuspended in Na^+ -Tyrode; the concentration of free Ca^{2+} was 0.8 mM and that of nigericin (Nig) was 0.015 μ g/ml. Tg (400 nM), PMA (50 nM) or ArA (8 μ M) was added at the arrow, followed by BSA (0.2 mg/ml; solid lines). Broken line, control trace showing the effect of BSA in the absence of ArA. (B) BCECF-loaded platelets were resuspended in Na^+ -Tyrode but EGTA was omitted. Where indicated, $LaCl_3$ was present at 20 μ M. The traces are representative of duplicate experiments with at least three different preparations with similar results.

In Figure 11 the decrease in pH_i was obtained with the electroneutral K^+/H^+ exchanger nigericin. Under these conditions the proton permeability of the plasma membrane is unaffected. Also in this situation, the activations of the exchanger by ArA, by the increase in $[Ca^{2+}]_i$ and by the activation of PKC are readily observable. In particular, the addition of ArA determines a further decrease in pH_i followed by a partial spontaneous recovery and by complete recovery after removal of the fatty acid with BSA. Under the conditions of Figure 11, the addition of BSA in the absence of ArA does not modify pH_i significantly.

Figure 11(B) shows that under these conditions La^{3+} can also substitute for BSA in promoting the recovery of pH_i in the presence of ArA.

The effect of ArA on the Na^+/H^+ exchanger is independent of an increased concentration of $[Ca^{2+}]_i$ or of the activation of PKC. This is shown in the experiment depicted in Figure 12, performed in BAPTA-AM-loaded platelets treated with the PKC inhibitor GF 109203X. In this situation of depressed $[Ca^{2+}]_i$ (by the presence of the internal Ca^{2+} chelator BAPTA), slightly higher concentrations of ArA are required for a strong activation of the exchanger. The initial decrease in pH_i was induced by nigericin; the subsequent addition of ArA induced a further decrease in pH_i followed by a partial spontaneous recovery, and finally BSA promoted a fast alkalinization. In the presence of NHA (Figure 12A, broken line), ArA induced a larger decrease in pH_i and the alkalinization after BSA was strongly decreased (after an initial rapid phase, probably representing the efflux of undissociated ArA). Control experiments (Figure 12B) showed that BSA alone added after nigericin did not induce the recovery of pH_i , and that BSA, when added before nigericin, decreased the acidification promoted by the ionophore and abolished the effects of ArA. Figure 12(C) shows a similar experiment, conducted with a higher concentration of nigericin. In this situation the nigericin-induced decrease of pH_i was about as large as that obtained in Figure 12(A) by the combined additions of nigericin and ArA. However, as also seen in Figure 12(C), BSA promoted a fast recovery of pH_i only when added after ArA. This experiment shows that the decrease of pH_i alone cannot account for the

Figure 12 The stimulation by ArA of the Na^+/H^+ exchanger is independent of Ca^{2+} and PKC

BCECF- and BAPTA- co-loaded platelets were resuspended in Na^+ -Tyrode. (A, B) Where indicated, GF 109203X (GF) was present at 2 μ M, nigericin (Nig) at 0.015 μ g/ml, ArA at 15 μ M, BSA at 0.4 mg/ml, saponin (Sap) at 200 μ g/ml and NHA (broken line) at 20 μ M. (C) The concentration of nigericin was 0.045 μ g/ml. The traces are representative of duplicate experiments with at least two different preparations with similar results.

strong activation of the Na^+/H^+ exchanger observed with ArA. Figure 12 also reports the final pH-equilibrating effect of saponin.

It can be concluded that when the pH_i is moderately decreased the activity of the Na^+/H^+ exchanger is not highly stimulated. It can be potentiated by known effectors such as the increase in $[Ca^{2+}]_i$ and the activation of PKC as well as by the newly described activator ArA.

DISCUSSION

It is known that the endomembrane Ca^{2+} -ATPase inhibitor Tg enables an increase in $[Ca^{2+}]_i$ in aspirinated human platelets in the absence of an agonist. In turn, the increased $[Ca^{2+}]_i$ has a complex effect on pH_i , which increases if the Na^+/H^+ exchanger is operative but decreases when the exchanger is inhibited or if the experiment is conducted in the absence of Na^+ ([24], and the present paper). As we show in the present study, BSA, which is without effect on $[Ca^{2+}]_i$, inhibits both the Tg-induced increase of pH_i and its decrease when the Na^+/H^+ exchanger is inhibited. Furthermore BSA loses its effect if it is pretreated with ArA. An effect similar to that of BSA is elicited by the phospholipase A_2 inhibitors OOPC and mepacrine. These observations led to the conclusion that the effect of Tg on pH_i in platelets might be mediated, at least in part, by ArA. Experiments were therefore performed to study the effect of exogenous ArA on platelet pH_i .

The first observation was that low concentrations of ArA (less than 10 μ M) activate a proton conductance pathway across the platelet plasma membrane, which allows H^+ movements in either direction, dictated by the existing driving force, the proton electrochemical potential ($\Delta p = \Delta \Psi + RT \ln [H^+]_o / [H^+]_i$). Thus under standard incubation conditions ArA induces H^+ influx, driven by the negative potential across the plasma membrane. The effect is potentiated by valinomycin, which hyperpolarizes the plasma membrane. In contrast, the suspension in a high- K^+ medium in the presence of the K^+ ionophore valinomycin (or the general cation ionophore gramicidin) and at high external pH is followed by H^+ efflux on addition of ArA. As might be expected, the effect of ArA is mimicked by low concentrations of the classic protonophore FCCP. However, ArA does not act as a protono-

phore as such [27–29]. This is because the phospholipid bilayer, which is permeable to the protonated free acid, is on the contrary impermeable to the anion. In fact, permeability to both species is required for a protonophoretic activity, where the combination of the fluxes of the free acid and the anion in opposite directions accounts for the movement of H^+ according to its electrochemical gradient. It was suggested recently that the influx of the undissociated free acid may also be assisted by a carrier protein [30].

A protonophoretic activity of fatty acids has been detected in isolated mitochondria. The effect is sensitive to the adenine nucleotide carrier inhibitors atractyloside, carboxyatractyloside and palmitoyl-CoA, and there is a consensus that the carrier itself mediates the transport of the fatty acid anion across the inner mitochondrial membrane [31,32]. Control experiments with atractyloside and carboxyatractyloside indicated that these agents have no effect on the ArA-induced acidification in platelets (L. Cavallini, M. Coassin, A. Borean and A. Alexandre, unpublished work). In the mitochondria from brown fat, the protonophoretic activity of the uncoupling protein thermogenin has recently been attributed to its operation as a fatty acid anion carrier [33]. At present, no data are available to discriminate whether ArA activates a true proton channel in the platelet plasma membrane or whether its protonophoretic action is to be ascribed to the presence of a fatty acid anion transporter.

An ArA-activated proton channel has been described and studied in detail in neutrophils and in macrophages [29,34,35]. The channel was reported to operate in association with the superoxide-anion-producing NADPH oxidase of these cells, and it is currently not clear whether it is an integral component of the oxidase [35] or an associated activity [36]. The finding that a similar protonophoretic activity is detectable in platelets that do not possess an NADPH oxidase opens the possibility that such an activity might be present in a larger variety of cells than previously suspected. To our knowledge this is the first observation of a plasma membrane-associated proton conductance pathway disjunct from the NADPH oxidase.

The platelet ArA-activated proton conductance pathway is highly sensitive to La^{3+} . It is less sensitive to Zn^{2+} (approx. 50% inhibition at 300 μM), a typical inhibitor of the proton conductance in neutrophils. It is unaffected by the protein kinase inhibitor staurosporine or by decreasing $[Ca^{2+}]_i$ with BAPTA.

The second finding in this study is that ArA, besides activating a proton conductance pathway, also acts as an activator of the Na^+/H^+ exchanger in platelets. The first indication for an effect of ArA on the antiporter was the observation that the increase of pH_i induced by Tg was partly sensitive to BSA and to the inhibitors of phospholipase A_2 , OOPC and mepacrine. However, no conclusions can easily be drawn from these experiments, because BSA, OOPC and mepacrine also affect the decrease in pH_i observed when the Na^+/H^+ exchanger is inhibited.

The activatory effect of ArA on the Na^+/H^+ antiporter is observed in experiments where first ArA is used to decrease pH_i (by activation of the proton conductance, which allows H^+ influx driven by the H^+ electrochemical potential; see above) and then it is removed with BSA. In these conditions the recovery of pH_i is fast. Being inhibited by NHA, it is probably mediated by the Na^+/H^+ exchanger. Interestingly, the recovery of pH_i on addition of BSA is much slower if FCCP rather than ArA is used to decrease pH_i . However, the BSA-induced alkalization becomes fast again if FCCP and ArA are present simultaneously. These experiments establish that the recovery of pH_i via the Na^+/H^+ antiporter is stimulated if platelets are treated with ArA before the removal of excess fatty acid with BSA. However, the activation of the antiporter by ArA does not require that the excess fatty acid be removed. In fact, the fast recovery of pH_i

previously decreased by ArA is also obtained, in the absence of BSA, if the ArA-activated proton conductance is inhibited with La^{3+} .

These results are consistent with the possibility that some derivative of ArA is the real activator of the Na^+/H^+ exchanger (although the effect of ArA is unmodified by inhibitors of the cyclo-oxygenase and lipoxygenase pathways). If ArA is the activator, the fast recovery of pH_i after BSA could indicate that the fatty acid is not removed readily from the exchanger or that the activation of the exchanger is lost only some time after the removal of ArA.

Similarly to other established activators of the Na^+/H^+ antiporter, such as the increase of $[Ca^{2+}]_i$ and the activation of PKC, ArA operates by increasing the activity of the exchanger when the pH_i is moderately decreased. Thus the decrease in pH_i obtained with nigericin or FCCP is partly or wholly reversed by Tg (which increases $[Ca^{2+}]_i$), by PMA (which activates PKC), or by ArA (once the H^+ conductance has been decreased, e.g. with La^{3+} or with BSA). The final set point of the antiporter is less markedly modified by these effectors.

The activation of the Na^+/H^+ exchanger by ArA is independent of Ca^{2+} and PKC, being visible in the presence of both the intracellular Ca^{2+} chelator BAPTA and the PKC inhibitor GF 109203X.

In conclusion, the present research has demonstrated that ArA, produced rapidly on platelet activation, contributes to the modulation of platelet pH_i in two ways, by activating a proton permeability pathway across the plasma membrane and by increasing the activity of the Na^+/H^+ exchanger when pH_i is decreased. The combined effect seems to be a stimulated influx of Na^+ , which probably contributes to osmotic control during activation.

This work was supported by 40% and 60% MURST funds, C. T. CNR no. 95.02102.CT04 and by Centro di Studio delle Biomembrane CNR.

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Received 9 February 1996/30 May 1996; accepted 25 June 1996