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The effect of BCECF on intracellular pH of human platelets

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Abstract

2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) is frequently used for fluorometric determination of intracellular pH (pH_i) and its metabolic changes. Studies of BCECF-loaded platelets have reported different pH_i values in the range of 6.98 to 7.35, despite the use of the same probe. It is now shown that intracellular BCECF ($BCECF_i$) content affects pH_i, and that its over-loading, leads to significantly lower pH_i. Different pH_i values can be reproduced by changing $BCECF_i$, as reflected by fluorescence intensity. The major loading factors are: the concentration of the probe parent compound, BCECF acetoxymethyl ester (AM), and whether this ester is partly hydrolyzed externally when applied in plasma. When least affected by BCECF, platelet pH_i is 7.34. High $BCECF_i$ does not affect ATP content, buffer capacity, activation of Na^+/H^+ exchange by protein kinase C (PKC) and basal PKC activity. On the other hand high $BCECF_i$ does inhibit the Na^+/H^+ exchange rate by over 50%. Since the Na^+/H^+ exchange strongly affects platelets pH_i, it is proposed that this inhibition accounts, at least partly, for the lowered pH_i in BCECF over-loaded platelets.

Keywords: BCECF; Intracellular pH; Human platelet; Na^+/H^+ exchange

1. Introduction

Maintenance of intracellular pH (pH_i) is a fundamental property of living cells, its regulation being an essential feature of cell homeostasis. Indeed, cellular metabolic processes are mostly sensitive to pH_i. Among the methods available for pH_i measurement, the utilization of spectroscopic probes generated in situ [1], is particularly effective and attractive. Studies of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-loaded platelets have reported different pH_i values in the range of 6.98 to 7.35 [2–9]. The plasma membrane Na^+/H^+ exchanger has a central role in pH_i homeostasis [2]. The Na^+/H^+ exchanger can be rapidly activated by protein kinase C (PKC)-dependent pathways [10]. Indeed, treatment of human platelets by direct PKC activator, such as phorbol 12-myristate 13-acetate (PMA), stimulates Na^+/H^+ exchange activity within seconds [11,12]. In the present study, we have attempted to elucidate the influence of procedural variables on the measured pH_i values. It is shown that pH_i of platelets, when least affected by BCECF, is about 7.34. In addition, platelet pH_i is significantly lowered upon overloading the platelets with fluorescent probe, due, at least partly, to an inhibition of the Na^+/H^+ exchange.

2. Materials and methods

2.1. Reagents

Albumin (bovine, fraction V), hirudin, *p*-(dipropylsulfamoyl)benzoic acid (probenecid), prostaglandin E_1 (PGE_1), Na propionate, amiloride, luciferase-luciferin and PMA were obtained from Sigma. BCECF acetoxymethyl ester (AM) was obtained from Molecular Probes. Sepharose 2B was purchased from Pharmacia. Triton X-100 (molecular grade) was obtained from International Biotechnologies, New Haven. [^{32}P]Phosphoric acid (9000 Ci/mmol) and [^{125}I]cAMP (2200 Ci/mmol) were obtained from

Abbreviations: pH_i, intracellular pH; BCECF AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; probenecid, *p*-(dipropylsulfamoyl)benzoic acid; PGE_1, prostaglandin E_1; PRP, platelet-rich plasma; OWP, once washed platelet; PPP, platelet-poor plasma; GFP, gel-filtered platelets; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; $BCECF_i$, intracellular BCECF

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Rotem Industries. Antibody against cAMP was obtained from Bio Makor.

2.2. Solutions

Acid-citrate-dextrose solution was composed of 65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate. The standard NaCl medium contained, in mM: 140 NaCl, 5 KCl, 0.42 NaH_2PO_4, 10 glucose and 20 Hepes, pH 6.8 or pH 7.35, as specified. *N*-methyl D-glucamine Cl medium was of the same composition, except that NaCl was replaced by *N*-methyl D-glucamine Cl. The NaCl-Na propionate medium contained in mM: 80 NaCl, 60 Na 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 ± 2 mosM with distilled water or the major salt. $1\times$ sample buffer contained: 3% SDS (w/v), 0.0015% Bromophenol blue (w/v), 5% 2-mercaptoethanol (v/v), 11% glycerol (v/v), 70 mM Tris-HCl, pH 6.8.

Stock solution of probenecid (0.1 M) and PGE_1 (2.8 mM) were prepared in ethanol, stock solution of BCECF AM (2.5 mg/ml) and PMA (10^{-3} M) were prepared in dimethylsulfoxide. Stock solution of luciferase-luciferin (40 mg/ml) was prepared in distilled sterile water.

2.3. Preparation of platelet suspension

Venous blood was drawn from healthy volunteers, aged 25–40 years, who had not received any medication during the previous 14 days. The blood was anticoagulated with acid-citrate-dextrose at a volume ratio of blood:anticoagulant of 6:1. Platelet-rich plasma (PRP) was obtained by centrifugation at $120 \times g$ for 10 min and had an initial pH of 6.5 ± 0.1. The pH rose to 6.8 within 30 min and remained stable at this pH. Once washed platelet (OWP) suspension was obtained by centrifugation of PRP at $1500 \times g$ for 10 min, removal of the platelet-poor plasma (PPP) and suspension of the cells in the standard NaCl medium, pH 6.8. For spectrofluorimetric measurements of pH changes, PRP or OWP suspension containing $2–3 \cdot 10^8$ platelets/ml were loaded in the dark with BCECF AM (7.3 μM final) for 30 min at 23°C or as specified. The suspensions were then gel-filtered through a Sepharose 2B column (10×0.76 cm). The solution, used to equilibrate the column and elute the platelets, was the standard NaCl medium. The suspensions of the gel-filtered platelets (GFP) were supplemented with $CaCl_2$ (1 mM), $MgCl_2$ (1 mM), hirudin (0.01 unit/ml) and probenecid (0.2 mM). Probenecid blocks organic anion transporters that remove fluorescent dyes from cytoplasmic matrix [13]. We found that the use of probenecid cuts down by 3-fold the leak of BCECF from platelets. Thus, most of the fluorescence measurements were conducted at $4.9 \pm 0.3\%$ leak ($n = 62$). The leak is taken as the fluorescence in the medium relative to the total in the suspension. All measurements were conducted within 50 min following gel filtration.

2.4. Determination of cytoplasmic pH changes

pH_i values and Na^+/H^+ exchange rates were determined, essentially, as described [3,11,12,14], but with some modifications. Fluorescence was measured in a Jasco FP-770 spectrofluorimeter, equipped with a mixing device, with wavelength settings at 495 and 525 nm for excitation and emission, respectively, using 5 and 10 nm slits, respectively. For measurement of pH_i changes an aliquot (10–20 μl) of GFP loaded with BCECF, was mixed with 1.8 ml of the standard NaCl medium, pH 7.35 ($1–2 \cdot 10^6$ platelets/ml) and fluorescence readings were taken for 6 s. For measurement of Na^+/H^+ exchange following intracellular acidification an aliquot was mixed with NaCl-Na propionate solution and fluorimetric tracings were recorded for 90 s, starting within < 3 s of adding the platelets. At the end Triton X-100 (9 μl of 10%) was added and calibration of pH versus 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 factor used to correct for the red shift of the intracellular dye was assessed [3,14]. The Na^+/H^+ exchange rate is expressed as ΔpH_i per 9 s at pH_i 7.0. In addition, Na^+/H^+ exchange was comparatively tested following acidification using another method [3]. GFP, suspended in *N*-methyl D-glucamine Cl medium, pH 6.8, were treated with 40 mM NH_4Cl for 18 min. GFP were then diluted 40-fold into an assay medium, composed like the Na propionate medium, but with NaCl fully substituting for the Na propionate.

2.5. Determination of the buffering capacity

The cytoplasmic buffering power was measured as described [3,15] by titration with NH_4^+. The pH_i of BCECF-loaded cells, suspended in *N*-methyl D-glucamine Cl medium, pH 7.35, was recorded, NH_4Cl (5 mM) was added and pH_i was recorded immediately. Buffering capacity was calculated as $\Delta[NH_4^+]_i/\Delta pH_i$. The concentration of NH_4^+ was calculated using pK value of 9.21 and assuming that the uncharged species (NH_3) is in equilibrium across the membrane.

2.6. Determination of intracellular ATP concentration

ATP was determined utilizing the luciferase-luciferin system, principally as described [16], with the following details. For extraction, 0.1 ml of OWP suspension was mixed with 2.9 ml of 0.04 M Tris borate buffer pH 9.2 and boiled for 5 min. Samples of 0.1 ml were assayed in 3 ml assay solution containing 6.7 mM NaH_2PO_4, 29.3 mM $MgSO_4$, 33.3 mM sodium arsenate and 1 mg luciferase-luciferin. Readings were conducted in a β-Counter.

2.7. Determination of intracellular cAMP concentration

Samples (0.7 ml PRP or OWP suspension) were centrifuged in a Jouan microfuge at $15000 \times g$ for 30 s. Platelets were resuspended in 0.1 ml of 50 mM Na acetate (pH 4.0) followed by heating at 100°C for 5 min. The supernatant obtained, after another centrifugation at $15000 \times g$ for 30 s, was used for cAMP determination by radioimmunoassay, principally as described [17], with the following details: Samples of the supernatant (0.1 ml) or cAMP standard solutions (1 to 500 pg) were incubated at 4°C for 24 h together with 0.005 ml of acetylation mixture (2:1 triethylamine/acetic anhydride), 0.1 ml anti cAMP antibodies (diluted 1:1000 with Tris buffer 44.6 mM, pH 7.4) and 0.1 ml [^{125}I]cAMP (20000 cpm/0.1 ml). At the end of the incubation, 0.1 ml of 50 mM Na acetate, pH 6.2, containing 1% albumin and 0.9 ml ethanol were added. After 10 min the samples were centrifuged at $700 \times g$ for 20 min at 4°C. The supernatant were discharged and the dry sediment was counted with γ-counter. The cAMP level in each sample was calculated according to the standard curve obtained.

2.8. Determination of PKC activity in intact human platelets

In order to radiolabel the ATP pool, platelets were resuspended in a phosphate free-standard NaCl medium and were incubated with 0.1 mCi/ml [^{32}P]phosphoric acid for 90 min at 37°C. After incubation, the platelets were gel-filtered, as described above, in order to remove excess [^{32}P]phosphoric acid. ^{32}P-radiolabeled platelets were mixed with $^{1}/_{4}$ volume of 5x concentrated sample buffer followed by heating at 100°C for 5 min. The samples were subjected to 11% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [18] and the 42 kDa protein band in each sample was identified by autoradiography, using Agfa RP2 x-ray films and DuPont lightning-Plus intensifying screens. The relative PKC activity in the corresponding protein bands was determined by scanning the films and integrating the peaks using the ImageQuant and Personal Densitometer Molecular Dynamics system.

3. Results

Studies of BCECF-loaded platelets have reported different pH_i values in the range of 6.98 to 7.35 [2–9]. The different pH_i values can be a result of procedural variables. One likely variable is the platelet suspension to which the probe parent compound, BCECF AM, is added: if added to PRP, as in Refs. [5,6] and, in part, [9], the reported pH_i is relatively high (≥ 7.28). However, if it is added to a washed platelet suspension, that has been treated by sedimentation and resuspension to remove the plasma, as in Refs. [2,4,7,8], then the pH_i is lower (≤ 7.15).

To test the role of the medium during BCECF loading, we determined pH_i while comparing two procedures: (a) BCECF AM was added directly to PRP, and (b) the platelets in PRP were first sedimented and suspended in the standard NaCl medium, pH 6.8, and then this OWP suspension was augmented with BCECF AM. In both procedures, the platelets were equally gel-filtered at the end of the probe loading period. Table 1 shows that the probe loading in the NaCl medium leads to a significantly lower pH_i than the loading in plasma, of the same external pH. Platelet aggregation, that might be associated with the preparation of OWP, can be excluded as a possible cause for the difference in pH_i for several reasons. Sedimentation of platelets from the anticoagulated PRP is conducted at low external pH that is not compatible with aggregation. Platelet aggregates were not visible in PRP, OWP suspension or GFP. Furthermore, the same pattern, shown in Table 1, is obtained whether or not PGE_1 is added to both media. PGE_1 was added to prevent aggregation [19] that may accompany sedimentation. cAMP was initially elevated by PGE_1, from the basal level of 2.08 pmol/10^8 platelets to 11.38 pmol/10^8 platelets but returned to the basal level following the gel filtration.

The difference in pH_i, due to the loading medium, decreases with time, after gel filtration. Upon incubation for 30 min at 23 or 37°C, the pH_i of the platelets loaded in NaCl is elevated by 0.07 ± 0.02 pH units. The pH_i change in the platelets loaded in plasma is significantly lower: -0.01 ± 0.02 units (mean ± S.E., $n = 5$, $P \leq 0.02$).

When the data summarized in Table 1, are analyzed with respect to the fluorescence intensity of the loaded platelets, pH_i values appear to be inversely correlated with the fluorescence (Fig. 1). Therefore, it is possible that pH_i is markedly affected by intracellular BCECF: the higher the concentration, the lower the recorded pH_i. If this possibility is correct, then the impact of the loading medium may actually result from the plasma esterase activity. During the loading period the esterase hydrolyzes BCECF AM yielding externally negatively charged BCECF, to which the membrane is profoundly less permeable, and thus the probe is less available internally.

The esterase activity can be followed fluorimetrically most effectively since, contrary to BCECF, the esterified probe, BCECF AM, emits virtually no fluorescence. To test the plasma esterase activity under the loading conditions, BCECF AM was added to PPP (containing no platelets), and to PRP (containing platelets). Time course of the change in fluorescence intensity was followed.

Table 1
Platelet pH_i as affected by the medium during BCECF loading

Property	Loading medium		P
	blood plasma	NaCl	
pH_i	7.38 ± 0.03	7.12 ± 0.03	< 0.001

$n = 18$; 9 in duplicate, 9 in triplicate.

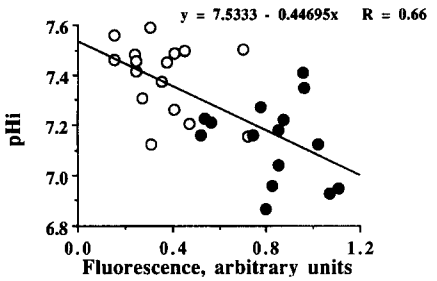

Fig. 1. Correlation between pH_i of BCECF-loaded platelets and the fluorescence intensity reflecting intracellular BCECF concentration. The data includes 18 different experiments. In each experiment, the open symbol refers to the loading of PRP while the solid symbol refers to the loading of OWP. The regression coefficient (0.66) is highly significant ($P < 0.001$).

PRP aliquots were assayed directly and after removal of the cells by rapid centrifugation. Fig. 2A shows that the plasma does indeed hydrolyze BCECF AM appreciably. The total fluorescence in PRP reflects the combined action of intracellular and plasma esterases. When the fluorescent intensity is normalized for the influence of pH, it is estimated that about 70% of BCECF AM molecules are hydrolyzed externally, by plasma esterases. Fig. 2B describes a parallel experiment, ran concomitantly, with OWP. BCECF AM was added to the platelet suspension and to two control media, namely, the supernatant of the

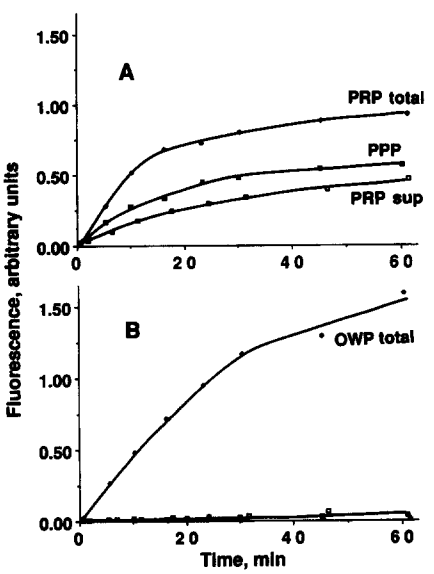

Fig. 2. Time course of hydrolysis of BCECF AM by platelet suspension and media as expressed by the change in fluorescence intensity. (A) BCECF AM was added to either PRP or PPP and aliquots (30 μl) were sampled at different times as indicated, and mixed with 1.77 ml of the NaCl medium, pH 6.8, for the fluorescence measurements. At the same time intervals PRP samples (60 μl) were also centrifuged in a Jouan microfuge at 15000× g for 30 s and the supernatant (30 μl) was similarly assayed (PRP sup). (B) BCECF AM was added to OWP (OWP total), to the supernatant obtained from OWP (1500× g, 10 min) and to the standard NaCl medium, pH 6.8. Sampling of cell-free aliquots of the platelet suspension and the fluorescence assays were as described above. The values of all fractions, except for OWP total were similarly minute. Additional details are given in Section 2.

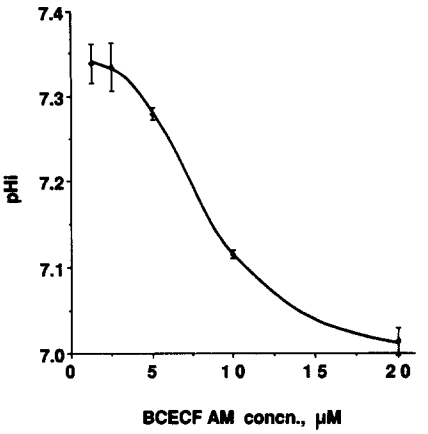

Fig. 3. Effect of BCECF AM concentration, added to OWP, on platelet pH_i. Shown are means (±S.E.) of triplicates. An additional experiment, with another blood donor, gave the same pattern.

OWP (to test for residual plasma esterase, comparable to PPP in Fig. 2A) and the standard NaCl medium, pH 6.8 (to test for spontaneous hydrolysis). Fig. 2B shows that the fluorescence in these control media, as well as in cell-free aliquots of the OWP suspension, are all negligible as compared with the marked fluorescence in the platelet suspension. Therefore, the external esterase activity of the OWP suspension is minute or nil and the fluorescence, of this suspension, results from intracellular hydrolytic activity.

To test, on this basis, the impact of intracellular BCECF without the influence of external esterase, a suspension of OWP was augmented with BCECF AM at varying concentrations from 1.25 to 20 μM. Fig. 3 shows a dependence of pH_i on added BCECF AM concentration. The pH_i is changing from 7.34 to 7.01 at the two concentration extremes. The platelet pH_i is least affected by the probe when BCECF AM concentration is 1.25 μM and the measured pH_i is 7.34.

The intracellular trapping of BCECF does not change due to the loading medium. The leak of BCECF from platelets loaded in NaCl is 5.7 ± 0.4% and the leak from platelets loaded in plasma is 5.3 ± 0.3% ($n = 7$).

Temperature and duration of loading are among the variables of the previous pH_i studies. To analyze their possible effect on pH_i, OWP suspension was incubated with 2 μM BCECF AM at 23 or 37°C, each for either 30 or 45 min. Upon loading at 23°C, pH_i is 7.39 ± 0.02 and 7.36 ± 0.03, after 30 and 45 min incubation, respectively. Upon loading at 37°C, pH_i is 7.18 ± 0.02 and 7.20 ± 0.01 for 30 and 45 min incubation periods, respectively. The differences with respect to duration (30 versus 45 min) at both temperatures are not significant, but they are highly significant ($n = 6$, $P < 0.01$) with respect to temperature (23 versus 37°C).

Loading thymocytes with 1 mM Quin 2 has been shown to lower ATP content by about 25% [20]. Therefore, we have examined the ATP content of OWP loaded with 2 or

10 μM BCECF AM. As shown in Table 2, the loaded cells indeed differed greatly (4-fold) in fluorescence intensity, reflecting a comparable difference in intracellular BCECF ($BCECF_i$) concentration, and also differed significantly in pH_i, similarly to the data shown above. Yet, ATP content of control platelets was found to be 3.06 ± 0.28 μmol/10^{11} platelets. No lowering by the probe loading at both concentrations could be detected. We also tested buffer capacity and Na^+/H^+ exchange, while comparing OWP loaded in the presence of either 2 or 10 μM BCECF AM. The buffer capacity was not modified. The values obtained (21–22 mmol · pH unit^{-1} · liter^{-1}) are close to those reported recently [8]. Unlike ATP content and buffer capacity, Na^+/H^+ exchange is affected by $BCECF_i$ content. The data in Table 2 show that, in conjunction with a higher fluorescence level, platelets, loaded with 10 μM BCECF AM, exhibit significantly lower Na^+/H^+ exchange rate. Furthermore, the same pattern of the impact of $BCECF_i$ on Na^+/H^+ exchange rate is observed, whether the rate is measured in Na propionate medium or by using NH_4^+-loaded platelets (data not shown).

As PKC activates Na^+/H^+ exchange in human platelets [11,12], it was interesting to test whether this activation is changing upon loading platelets with 2 or 10 μM BCECF AM. PMA was used as specific activator of PKC and the results show that PMA activates Na^+/H^+ exchange rate by 2.8 ± 0.8-fold in 2 μM BCECF AM loaded platelets ($n = 3$, each in duplicate). No lowering by the probe loading at 10 μM could be detected (Na^+/H^+ exchange rate was activated by 3.1 ± 0.8-fold).

The signal, from PKC to the Na^+/H^+ exchanger, is equally transmitted in 2 and 10 μM BCECF AM loaded OWP. Therefore, it was interesting to test whether basal PKC activity was changed upon loading platelets with BCECF AM. Phosphorylation of the 42 kDa protein in platelets was measured, as this protein has been shown to be a substrate of PKC and its phosphorylation correlates directly with PKC activation [21–24]. The phosphorylation levels of the 42 kDa protein, in 0, 2 and 10 μM BCECF AM loaded OWP platelets, were quantified by densitometric integration of autoradiograms. The ratio of 2 μM BCECF AM loaded OWP to control phosphorylation averaged 1.03 ± 0.04 ($n = 4$). The ratio of 10 μM BCECF AM loaded OWP to control phosphorylation averaged 0.97 ± 0.10 ($n = 4$).

4. Discussion

BCECF is frequently used for fluorimetric determination of pH_i and its metabolic changes. We conclude that the pH_i of BCECF-loaded platelets is affected by the probe and that this probe lowers the pH_i significantly. Several lines of evidence support this conclusion: (a) When platelets are loaded with BCECF while suspended in the plasma, a major portion of BCECF AM is hydrolyzed externally (Fig. 2A). Consequently, as shown in Table 1, the pH_i of PRP is significantly higher than that of OWP, which are loaded in a medium with a low esterase activity (Fig. 2B). (b) The pH_i of BCECF-loaded platelets is inversely correlated with the intracellular BCECF concentration, as reflected by the cells fluorescence (Fig. 1). (c) OWP are affected by the BCECF AM concentration during loading, with a marked dependence at a concentration of ≥ 3 μM (Fig. 3). These observations would explain the extensive variance in pH_i of BCECF-loaded platelets, as reported in different studies. Generally, studies employing conditions that increase intracellular BCECF concentration report a relatively lower pH_i, while restricted loading leads to higher pH_i. The determinant loading factors are: (a) BCECF AM concentration, (b) the medium esterase activity and (c) the loading temperature. In addition, the media used to suspend the BCECF-loaded platelets can also affect pH_i, as reported by Sage et al. [9]. Noticeable is a pH_i of 7.02 in HCO_3^- medium and a pH_i of 7.28 in 23 mM K^+/Hepes medium. It is not known whether Na^+/H^+ exchange rates are affected by these different media.

The reasons for the BCECF-effect on pH_i require further clarification. BCECF is a highly charged molecule and therefore it is conceivable that its increased concentration in the platelets might affect properties that can influence pH_i. Therefore, we tested, in platelets treated with low and high intracellular concentrations of BCECF, the ATP con-

Table 2
Platelet pH_i, buffer capacity and Na^+/H^+ exchange as affected by external BCECF AM concentrations

Property	BCECF AM concentration (μM)		P
	2	10	
Platelet fluorescence arbitrary units	0.30 ± 0.04	1.19 ± 0.12	< 0.001
pH_i	7.32 ± 0.02	7.07 ± 0.08	< 0.02
ATP content μmol/10^{11} platelets	3.35 ± 0.30	3.01 ± 0.33	NS
Buffer capacity mmol · pH unit^{-1} · liter^{-1}	20.6 ± 2.0	22.2 ± 1.5	NS
Na^+/H^+ exchange rate $\Delta pH/9$ s (at pH_i 7.0)	0.034 ± 0.005	0.014 ± 0.002	< 0.01

$n = 5$, with 3 or more replicates; NS, not significant.

tent, the buffer capacity and the Na^+/H^+ exchange. ATP content and buffer capacity were not modified while Na^+/H^+ exchange was affected by $BCECF_i$ content. The data in Table 2 show that in conjunction with a higher fluorescence level due to platelet loading with higher BCECF concentration, platelets exhibit significantly lower Na^+/H^+ exchange rate.

How does BCECF affect Na^+/H^+ exchange rate? PMA, an activator of PKC, added at saturating concentration, stimulates the Na^+/H^+ exchange rates essentially as described earlier [11,12]. However, it does not alleviate the relative difference in exchange rate between platelets with low and high intracellular concentrations of BCECF. Furthermore, basal PKC activity was not affected by the probe loading. Therefore, the reduced Na^+/H^+ exchange activity is apparently not caused by lowered PKC activity in platelets loaded with high intracellular concentrations of BCECF. Damage to the Na^+/H^+ exchanger by other routes, such as proteolysis has yet to be examined.

It is possible that the bulky and negatively charged BCECF molecule interferes, at high concentration, with the unique allosteric site of the Na^+/H^+ exchanger, activated by H^+ [10,25]. If BCECF charge plays a role in this interference (e.g., by competition with the allosteric site for H^+), it is likely to be a localized effect, as the overall buffer capacity is not changed by the probe over-loading (Table 2).

The question, whether the effect of BCECF AM loading on pH_i is due to BCECF itself or due to the hydrolysis products of the AM ester, remains to be answered.

Na^+/H^+ exchange plays a major role in pH_i regulation in platelets. The pH_i of BCECF-loaded platelets, when least affected by the probe (Fig. 3), is about 7.34. Overloading with BCECF lowers the pH_i of platelets at least in part due to reduction in the Na^+/H^+ exchange activity. The differences in $BCECF_i$ may thus lead to artificial differences in the measured exchange rates and pH_i values, not only in platelets but in other cells as well. In addition to Na^+/H^+ exchange, the intracellular hydrolysis of BCECF AM could lead to lowering of pH_i in case of over loading, although the buffer capacity is not significantly modified.

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