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Mechanism of Collagen Activation in Human Platelets*

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The mechanism of collagen-induced human platelet activation was examined using Ca^{2+}, Na^+, and the pH-sensitive fluorescent dyes calcium green/fura red, sodium-binding benzofuran isophthalate, and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein. Administration of a moderate dose of collagen (10 μg/ml) to human platelets resulted in an increase in $[Ca^{2+}]_i$ and platelet aggregation. The majority of this increase in $[Ca^{2+}]_i$ resulted from the influx of calcium from the extracellular milieu via the Na^+/Ca^{2+} exchanger (NCX) functioning in the reverse mode and was reduced in a dose-dependent manner by the NCX inhibitors 5-(4-chlorobenzyl)-2′,4′-dimethylbenzamil (KD_{50} = 4.7 ± 1.1 μM) and KB-R7943 (KD_{50} = 35.1 ± 4.8 μM). Collagen-induced platelet aggregation was dependent on an increase in $[Ca^{2+}]_i$ and could be inhibited by chelation of intra- and extracellular calcium through the administration of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) and EGTA, respectively, or via the administration of BAPTA-AM to platelets suspended in no-Na^+/HEPES buffer. Collagen induced an increase in $[Na^+]_i$ (23.2 ± 7.6 mM) via the actions of thromboxane A_2 and, to a lesser extent, of the Na^+/H^+ exchanger. This study demonstrates that the collagen-induced increase in $[Ca^{2+}]_i$ is dependent on the concentration of Na^+ in the extracellular milieu, indicating that the collagen-induced increase in $[Na^+]_i$ causes the reversal of the NCX, ultimately resulting in an increase in $[Ca^{2+}]_i$ and platelet aggregation.

Collagen is the most thrombogenic component of the subendothelium (1). Following vascular damage, collagen is exposed to circulating platelets and both acts as a substrate for the adhesion of platelets (2–4) and induces platelet activation (4). The prevailing evidence proposes that two receptors are involved in the platelet response to collagen; integrin $\alpha_2\beta_1$ acts to adhere platelets to collagen, allowing platelets to interact with the lower affinity receptor glycoprotein VI, which is mainly responsible for platelet activation (3, 5).

Many of the platelet responses to collagen progress simultaneously when platelets adhere to collagen. At high concentrations, collagen activation of platelets has been shown to proceed through activation of phospholipase Cγ2 and subsequent cleavage of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (6, 7). Inositol 1,4,5-trisphosphate induces the release of calcium from the dense tubular system (8, 9), whereas 1,2-diacylglycerol activates protein kinase C (10). The collagen-induced inositol 1,4,5-trisphosphate-mediated increase in $[Ca^{2+}]_i$ is accompanied by an influx of calcium from the extracellular milieu (11, 12). 1,2-Diacylglycerol and calcium mediate the characteristic platelet activation responses such as shape change, granule secretion, and aggregation.

At lower concentrations, many of the effects of collagen are enhanced by its production of thromboxane A_2 (TXA)[1] (6, 13–15). The collagen-induced increase in $[Ca^{2+}]_i$ can be decreased by inhibiting the production of TXA via the pretreatment of platelets with cyclooxygenase inhibitors such as aspirin (11, 16, 17).

Calcium is an important second messenger in the platelet activation cascade. At rest, a $[Ca^{2+}]_i$ of ~100 nM is maintained by a balance between the leak of Ca^{2+} into the platelet and the concurrent efflux of free Ca^{2+} across the plasma membrane of the platelet and accumulation in intracellular stores (18, 19). Ca^{2+} is moved out across the plasma membrane through the actions of the plasma membrane Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger (NCX). Plasma membrane Ca^{2+}-ATPases are membrane-inserted enzymes that use the energy of ATP hydrolysis to move Ca^{2+} against its gradient and across the membrane. The NCX is capable of moving Ca^{2+} into or out of the platelet cytosol in exchange for Na^+ (20, 21). In the resting state, the NCX removes Ca^{2+} from the platelet cytosol. Internally, Ca^{2+} is transported into the dense tubular system by the sarco/endoplasmic reticulum Ca^{2+}-ATPases 2b and 3 (22, 23).

In response to a moderate dose of collagen (10 μg/ml), ~70% of the increase in $[Ca^{2+}]_i$ is due to the influx of Ca^{2+} from the extracellular milieu, with the remainder as a function of Ca^{2+} release from the dense tubular system (12). Because voltage-gated calcium channels are not present in platelets (24–27), either a receptor-operated calcium channel or a reverse mode NCX could contribute to the influx of Ca^{2+} (20, 21, 28). The initial influx of Na^+ would be essential for the reversal of NCX.

Influx of Na^+ has been shown upon activation of platelets by thrombin, ADP, and polylysine (29–31). Matsuoka and Hilgemann (33) reported that at a $[Ca^{2+}]_i$ of 100 nM and a membrane potential of −60 mV (platelet membrane potential at rest and in response to collagen (32)), a small change in $[Na^+]_i$ can result in the reversal of the NCX. Na^+/H^+ exchangers (NHEs) could also contribute to the increase in $[Na^+]_i$ and subsequent NCX reversal.

The objective of this study was to determine the mechanism responsible for the collagen-induced increase in human platelet

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[1] The abbreviations used are: TXA, thromboxane A_2; NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+ exchanger; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); CBDMB, 5-(4-chlorobenzyl)-2′,4′-dimethylbenzamil; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride.

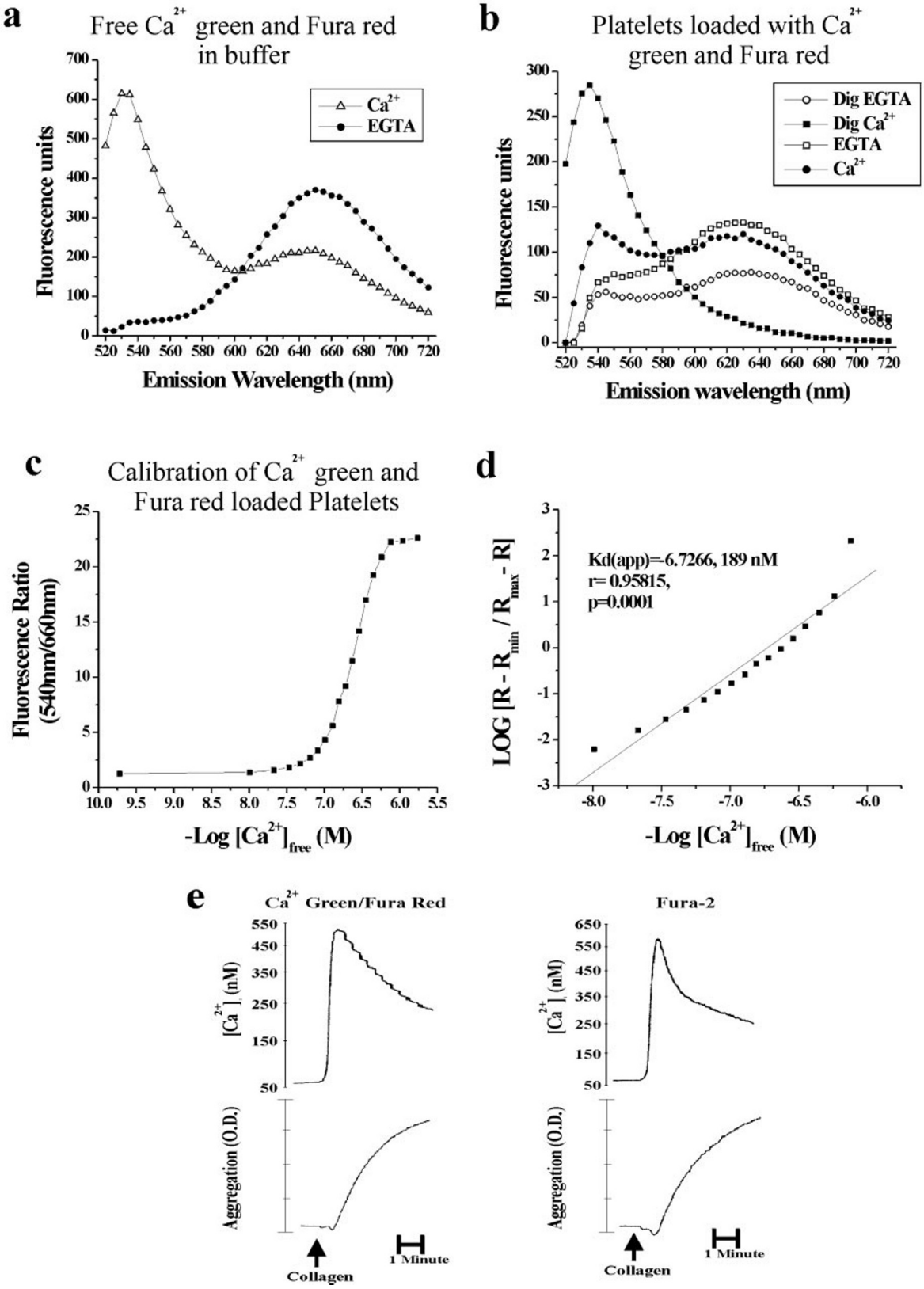

FIG. 1. **Validation of the two-dye system used for $[Ca^{2+}]_i$ measurements at 37 °C.** The emission scans were obtained with a SPECTRAmax Gemini EM spectrofluorometer (Molecular Devices, Sunnyvale, CA), and all the other data were obtained with a Jasco Model CAF-110 ion analyzer. *a*, emission scan of the free dye mixture (25 nM calcium green and 22 μM fura red) in the presence of 1 mM calcium or 10 mM EGTA added to HEPES buffer. *b*, emission scan of a suspension of platelets that were loaded with the two dyes with and without permeabilization with 0.2 mM digitonin (*Dig*). There was a leftward shift in the fura red peak by 20 nm in intact platelets compared with that in buffer solution. *c*, concentration-response curve of the ratio of fluorescence at 540/660 nm from dyes in permeabilized platelets plotted against the negative log of the free calcium concentration. Free calcium was varied with 10 mM EGTA buffers. *d*, calculation of the apparent K_d using the ratio changes in one batch of permeabilized platelets. The mean $K_{d(app)}$ from eight different batches of platelets was 188 ± 7. *e*, typical traces of the collagen (10 μg/ml)-induced change in $[Ca^{2+}]_i$ and aggregation measured with the single ratiometric dye (Fura-2; *right panel*) and the two-dye system (calcium green and fura red; *left panel*) used for ratiometry.

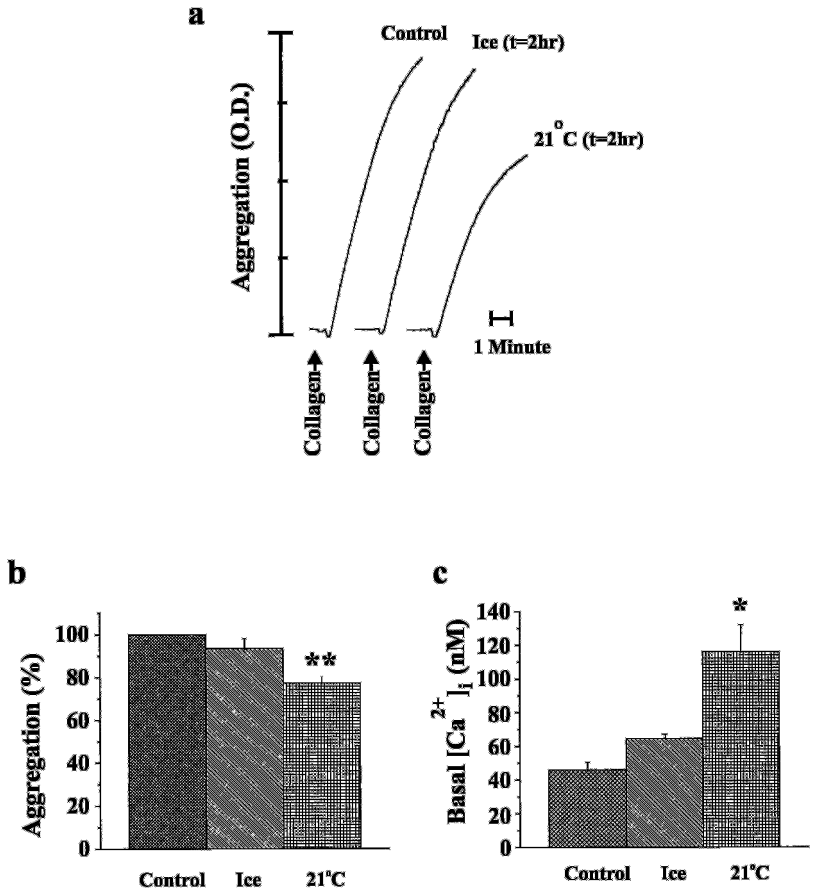

FIG. 2. **Filtered platelet response to storage.** Platelets suspended in calcium-free HEPES buffer were stored on ice or at room temperature (21 °C) for 2 h. Aliquots of the platelets were then incubated with 1 mM Ca^{2+} at 37 °C for at least 5 min prior to measurement of their collagen-induced aggregation and basal $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ and collagen-induced aggregation were measured for freshly isolated platelets (t = 0 h) as a control. *a*, typical traces of platelet function as determined by collagen (10 μg/ml)-induced platelet aggregation for samples stored either on ice or at room temperature (21 °C) for 2 h compared with freshly isolated platelets (*Control*). *b*, percent change in collagen-induced platelet aggregation relative to t = 0 h control measured 2 min following the addition of 10 μg/ml collagen to platelets stored for 2 h either on ice or at room temperature (21 °C). *c*, average basal $[Ca^{2+}]_i$ in freshly isolated platelets and platelets that had been stored for 2 h in calcium-free HEPES buffer on ice or at room temperature (21 °C). *, $p < 0.05$ (n = 5); **, $p < 0.005$ (n = 5).

$[Ca^{2+}]_i$. Our findings demonstrate that the majority of the collagen-induced increase in $[Ca^{2+}]_i$ results from the influx of Ca^{2+} from the extracellular milieu via the NCX functioning in the reverse mode. The reverse mode NCX appears to be a result of the influx of Na^+ into the platelet cytosol via the actions of TXA and, to a lesser extent, of the NHE.

MATERIALS AND METHODS

Platelet Isolation—Venous blood (40 ml) was drawn from healthy volunteers, who denied taking aspirin for at least 14 days prior to participation, into tubes containing the anticoagulant EDTA. The platelet-rich plasma was isolated from blood samples by centrifugation at 600 rpm for 15 min. Platelets were isolated from platelet-rich plasma by centrifugation at 2000 rpm for 15 min. Platelet samples were resuspended in 500 μl of platelet-poor plasma and loaded with the appropriate fluorescent dye.

Fura-2 could not be used for the determination of $[Ca^{2+}]_i$ due to interactions with a number of the pharmacological compounds required for evaluating the role of the NCX (34). In this study, two dyes, calcium green-AM dye (10 μM) and fura red-AM dye (20 μM), which are excited at long wavelengths, but have opposite responses in emission upon binding calcium, were used in combination. Calcium green and fura red have single peaks of excitation or emission and are difficult to work with alone, as artifacts introduced from platelet shape change, adhesion, dye loading, and platelet number affect fluorescence readings. With the ratiometric approach of combining two dyes, these artifacts were avoided, and the calculated values became very reproducible in different batches of platelets. This combination of dyes has been used in many tissues, including platelets (35). This approach was validated in a series of experiments shown in Fig. 1. The emission spectrum of the free dye combination within in platelets (Fig. 1*b*) was similar to that in buffer solution (Fig. 1*a*). There was only one component in the dose-response relationship of the fluorescence ratio with free $[Ca^{2+}]_i$ in digitonin-permeabilized platelets. The free calcium concentration was calculated as described (36) and takes into account the presence of 1 mM $MgCl_2$ in the assay buffer and the pH of the solution. The apparent binding constant ($K_{d(app)}$) of the dye combination for calcium calculated from a batch of platelets as shown in Fig. 1*d*. The mean $K_{d(app)}$ from eight different platelet samples was 188 ± 7. The time course of the collagen-induced release of calcium from platelets with the calcium green/fura red combination was very similar to that observed with Fura-2, a ratiometric dye widely used for calcium measurements in platelets (Fig. 1*e*).

pH_i was determined with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM dye (10 μM), and $[Na^+]_i$ was measured with sodium-binding benzofuran isophthalate-AM dye (20 μM). Each sample was incubated for 1 h at 37 °C to load the dye.

Preparation of Platelet Samples—Following incubation, the platelets were separated from the plasma and extracellular dye by gel filtration using a Sepharose CL-2B column. The platelets were eluted in Ca^{2+}-free HEPES buffer containing 140 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 5.5 mM glucose, 0.25% albumin, and 20 mM HEPES (pH 7.4); counted in a Coulter counter; and adjusted to 2×10^8 platelets/ml. Where required, *N*-methylglucamine or choline chloride was substituted for NaCl to maintain equal osmolarity. Prior to each experiment, 1 mM Ca^{2+} was added to the platelet suspension.

Where appropriate, a sample of the platelet suspension was incubated in 0.5 mM aspirin for 1 h at 37 °C. The sample was then centrifuged to isolate the platelets, which were resuspended in the appropriate volume of calcium-free HEPES buffer to maintain a platelet concentration of 2×10^8 platelets/ml.

Previous studies have shown that isolated plasma-free platelets stored at 4 °C retain their functions for several hour (37–40). Fig. 2 shows that, under these conditions, platelets retained a similar basal $[Ca^{2+}]_i$ and function (aggregation) compared with those of freshly isolated platelets for up to 2 h. In contrast, platelets incubated at room temperature (21 °C) in calcium-free buffer deteriorated rapidly; and after 2 h, there was a significant increase in basal $[Ca^{2+}]_i$ and a reduction in platelet function (aggregation) (Fig. 2). Storage of platelets in calcium-free buffer on ice or at room temperature had no significant effect on the collagen-induced change in $[Ca^{2+}]_i$ (299.8 ± 11.1 nM (n = 5; not significant) and 363.3 ± 53.1 (n = 5; not significant), respectively) compared with the control (311.1 ± 33.7, n = 5) measured 2 min following the addition of collagen (10 μg/ml). Therefore, platelets in calcium-free buffer were stored on ice throughout the course of this study.

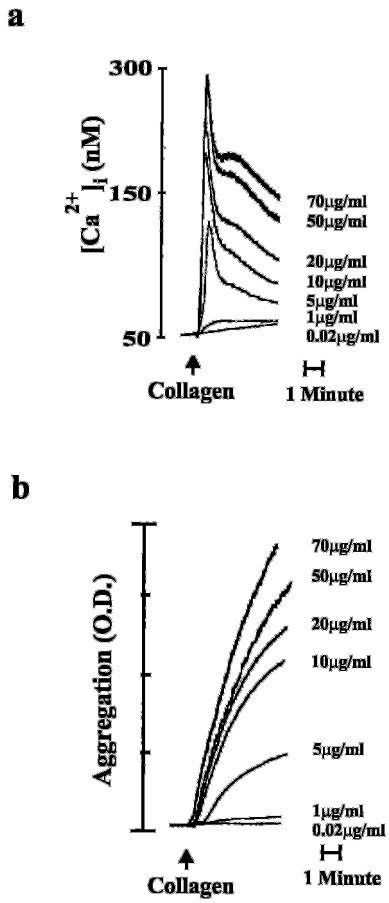

FIG. 3. **Collagen dose response.** *a*, typical superimposed traces of the collagen-induced change in $[Ca^{2+}]_i$ in a sample of platelets administered increasing doses of collagen; *b*, the corresponding effect on platelet aggregation.

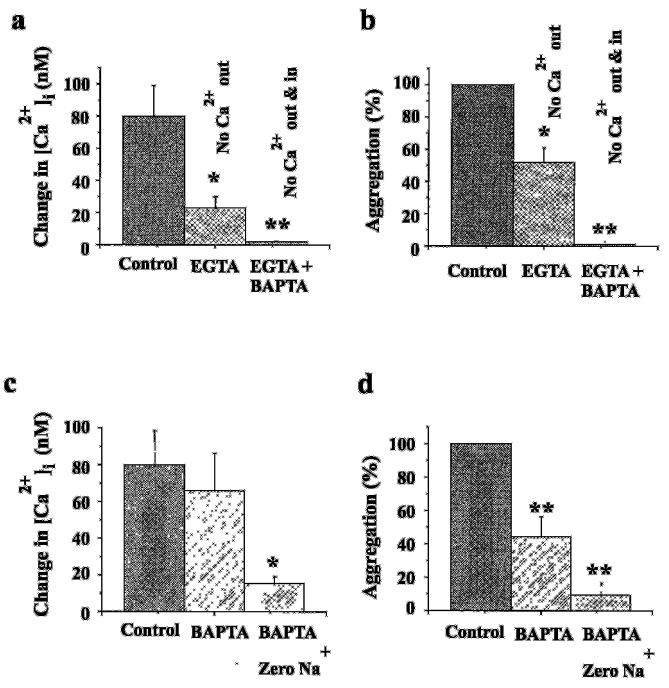

FIG. 4. **Role of sodium and calcium in collagen activation of human platelets.** *a*, collagen-induced change in $[Ca^{2+}]_i$ measured 2 min following the administration of collagen (10 μg/ml) for platelet samples suspended in 1 mM Ca^{2+} (*Control*), 0 mM extracellular calcium achieved by administering 5 mM EGTA 1 min prior to the addition of collagen (10 μg/ml), or total 0 mM calcium achieved through the preincubation of platelet samples with BAPTA-AM (20 μM) and the administration of 5 mM EGTA 1 min prior to the addition of collagen (10 μg/ml). *b*, relative percent change in the collagen-induced platelet aggregation of platelet samples administered 5 mM EGTA 1 min prior to the administration of collagen or pretreated with BAPTA-AM (20 μM) and administered 5 mM EGTA 1 min prior to the administration of collagen compared with platelet samples suspended in 1 mM Ca^{2+}/HEPES buffer (*Control*). Platelet aggregation was measured 2 min following the addition of collagen (10 μg/ml). *c*, collagen-induced change in $[Ca^{2+}]_i$ measured 2 min following the administration of 10 μg/ml collagen to platelets suspended in 1 mM Ca^{2+}/HEPES buffer containing 140 mM Na^+ (*Control*) and to platelets pretreated with 20 μM BAPTA-AM and suspended in 1 mM Ca^{2+}/HEPES buffer containing either 140 mM Na^+ (*BAPTA*) or 0 mM Na^+ (*BAPTA Zero Na*$^+$). *d*, relative change in collagen-induced platelet aggregation measured 2 min following the administration of collagen for platelet samples pretreated with 20 μM BAPTA-AM and suspended in 1 mM Ca^{2+}/HEPES buffer containing either 140 mM Na^+ (*BAPTA*) or 0 mM Na^+ (*BAPTA Zero Na*$^+$) compared with the control. *, $p < 0.05$ ($n = 4$); **, $p < 0.005$ ($n = 4$).

Fluorescence and Aggregation Measurement—Aliquots of the platelets were incubated with 1 mM Ca^{2+} at 37 °C for at least 5 min prior to conducting the test response. Changes in fluorescence and aggregation were simultaneously measured at 37 °C in a Jasco Model CAF-110 ion analyzer. The excitation wavelength for $[Ca^{2+}]_i$ measurement was 500 nm, and the emission wavelengths were 540 and 660 nm. The excitation wavelengths for $[pH]_i$ measurement were 440 and 500 nm, and the emission wavelength was 540 nm. For $[Na^+]_i$ measurements, the excitation wavelengths were 340 and 380 nm, and the emission wavelength was 500 nm. $[Ca^{2+}]_i$, $[pH]_i$, and $[Na^+]_i$ were calculated according to previously published formulas (39, 41). Aggregation was measured as a change in absorbance using a near-infrared LED 950-nm light source.

Chemicals—Calcium green-AM, fura red-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-AM, sodium-binding benzofuran isophthalate-AM, and BAPTA-AM were purchased from Molecular Probes, Inc. (Eugene, OR) and dissolved in Me_2SO. 5-(4-Chlorobenzyl)-2′,4′-dimethylbenzamil (CBDMB) was obtained from Dr. E. J. Cragoe, Jr. and dissolved in Me_2SO. KB-R7943 (2-[2-(4,4-nitrobenzyloxy)phenyl)-ethyl]isothiourea) was purchased from Tocris Cookson Ltd. and dissolved in Me_2SO. 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) was obtained from Dr. E. J. Cragoe, Jr. and dissolved in Me_2SO. Collagen was obtained from Nycomed Arzneimittel (Munich, Germany). Phorbol 12,13-dibutyrate (Sigma), and U-46619 (Cayman Chemical Co., Inc.) were dissolved in Me_2SO. Aspirin was obtained from Sigma and dissolved in Me_2SO. Sepharose CL-2B was obtained from Amersham Biosciences. All other chemicals were purchased from Sigma.

Statistical Analysis—All data are expressed as means ± S.E.; *n* denotes the number of participants from which platelets were obtained. Student's paired *t* test was used; and where applicable, analysis of variance was used for blocked comparisons. $p < 0.05$ was taken as significant.

RESULTS

Calcium Requirement for Collagen-induced Platelet Activation—Administration of collagen to human platelets suspended in 1 mM Ca^{2+} resulted in a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 3*a*) (11). The KD_{50} for the collagen-induced increase in $[Ca^{2+}]_i$ measured 1 min following the administration was 10.1 ± 2.2 μg/ml ($n = 6$). To determine the absolute requirement of $[Ca^{2+}]_i$, platelets were suspended in Ca^{2+}-free medium with 5 mM EGTA 1 min prior to their activation with collagen (10 μg/ml) or loaded with 20 μM BAPTA-AM at 37 °C for 30 min and administered 5 mM EGTA 1 min prior to their activation with collagen (10 μg/ml). The chelation of extracellular calcium with EGTA decreased the collagen-induced change in $[Ca^{2+}]_i$ (22.99 ± 6.99 nM, $n = 4$; $p < 0.05$) and aggregation (51.77 ± 9.14%, $n = 4$; $p < 0.05$). In the case of BAPTA and EGTA, collagen induced neither a change in $[Ca^{2+}]_i$ (1.82 ± 0.53 nM, $n = 4$; $p < 0.005$) (Fig. 4*a*) nor aggregation (1.00 ± 1.88%, $n = 4$; $p < 0.005$) (Fig. 4*b*) compared with platelets that were untreated with BAPTA-AM and EGTA.

Effect of Extracellular Sodium on the Collagen-induced Increase in $[Ca^{2+}]_i$—To determine the effects of external Na^+ on the collagen-induced increase in $[Ca^{2+}]_i$, platelets were sus-

TABLE I

Effect of extracellular sodium on the collagen-induced increase in cytosolic Ca^{2+} and aggregation

The collagen-induced change in $[Ca^{2+}]$, and aggregation was measured 2 min following the administration of 10 μg/ml collagen. Platelets were suspended in HEPES buffer containing 140 (control), 28, or 0 mM Na^+. Equal isomolarity was achieved by the substitution of either choline chloride or *N*-methylglucamine in the reduced Na^+/HEPES buffers.

Treatment	$\Delta[Ca^{2+}]_i$	Sample size	Aggregation	Sample size
	nM		%	
140 mM Na^+ (control)	329.1 ± 28.3	4	100	4
28 mM Na^+, 112 mM choline chloride	300.1 ± 28.5[a]	4	70.1 ± 7.1[b]	4
28 mM Na^+, 112 mM *N*-methylglucamine	270.7 ± 26.1[a]	4	68.7 ± 3.5[b]	4
0 mM Na^+, 140 mM choline chloride	209.4 ± 32.6[b]	4	63.9 ± 4.4[c]	4
0 mM Na^+, 140 mM *N*-methylglucamine	215.3 ± 12.6[b]	4	66.5 ± 4.1[c]	4

[a] Not significant.
[b] *, $p < 0.05$ ($n = 4$).
[c] **, $p < 0.005$ ($n = 4$).

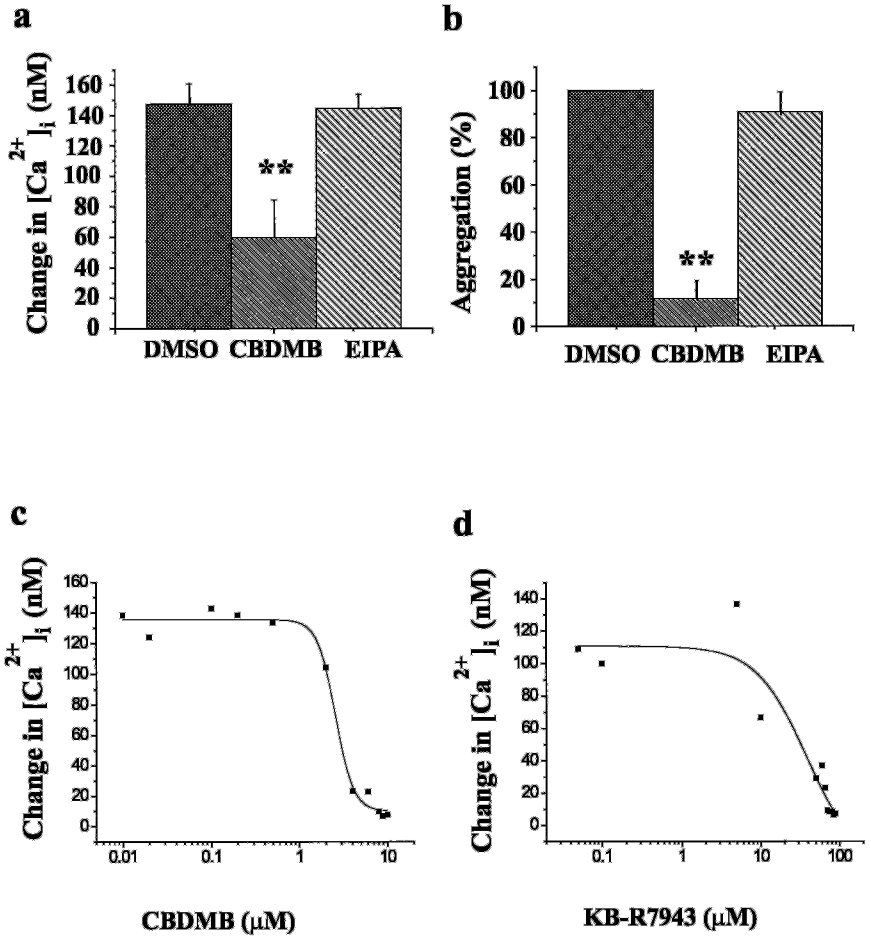

FIG. 5. **Role of the NCX and NHE in collagen activation of human platelets.** *a*, collagen-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of collagen (10 μg/ml) to platelets suspended in 1 mM Ca^{2+}/HEPES buffer and administered Me_2SO (*DMSO*; control vehicle), CBDMB (4 μM), or EIPA (50 μM) 3 min prior to the addition of collagen. *b*, relative percent change in collagen-induced platelet aggregation measured 2 min following the administration of collagen (10 μg/ml) to platelets administered CBDMB (4 μM) or EIPA (50 μM) 3 min prior to the addition of collagen compared with the control. *c* and *d*, typical dose-response curves for the effects of the NCX blockers CBDMB and KB-R7943, respectively, on the collagen-induced change in $[Ca^{2+}]_i$ measured 30 s following the administration of collagen (10 μg/ml). **, $p < 0.005$ ($n = 5$).

pended in buffer containing 140, 28 (low Na^+), or 0 (no Na^+) mM Na^+. Low-Na^+/HEPES and no-Na^+/HEPES buffers contained either *N*-methylglucamine or choline chloride to maintain equal osmolarity. Removal of extracellular Na^+ resulted in a significant reduction in both $[Ca^{2+}]_i$ and aggregation regardless of the substituent used to maintain iso-osmolality (Table I). To ensure that these results were due to the absence of Na^+ and not to the effects of *N*-methylglucamine or choline chloride, in a separate series of experiments, the no-extracellular Na^+ experiment was repeated using iso-osmolar sucrose (280 mM). The collagen-induced change in $[Ca^{2+}]_i$ measured 2 min following the addition of 10 μg/ml collagen was reduced by 61.2 ± 5.3% ($n = 5$; $p < 0.0005$) for platelets suspended in no-Na^+/sucrose-substituted buffer. Platelets suspended in no-Na^+/choline chloride- or no-Na^+/*N*-methylglucamine-substituted buffer had a collagen-induced change in $[Ca^{2+}]_i$ that was reduced by 35.4 ± 11.8% ($n = 4$; $p < 0.05$) and 33.4 ± 5.1% ($n = 4$; $p < 0.05$), respectively. From these results, we concluded that the reduction in collagen-induced activation of human platelets in the absence of extracellular Na^+ is due to the effect of Na^+ and not to the substituent used to replace it.

Chelation of intracellular calcium by BAPTA alone in medium containing 1 mM Ca^{2+} had no effect on the collagen-induced change in $[Ca^{2+}]_i$ (65.9 ± 20.4 nM, $n = 4$; not significant) (Fig. 4*c*), but reduced collagen-induced aggregation (55.7 ± 12.0%, $n = 4$; $p < 0.005$) (Fig. 4*d*). In contrast, chelation of intracellular calcium in platelets suspended in no-Na^+ medium containing *N*-methylglucamine reduced the collagen-induced increase in $[Ca^{2+}]_i$ by 64.4 ± 3.6 nM ($n = 4$; $p < 0.005$) (Fig. 4*c*) and the collagen-induced aggregation by 90.6 ± 7.3% ($n = 4$; $p < 0.005$) (Fig. 4*d*).

Inhibition of the NCX by CBDMB caused a significant de-

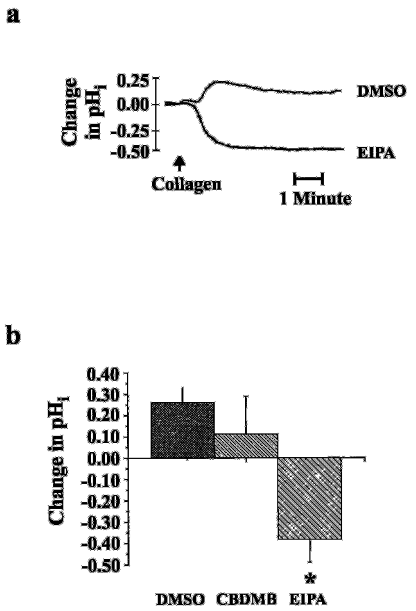

FIG. 6. **pH$_i$ response of collagen-activated platelets.** *a*, typical superimposed traces of the collagen-induced change in pH$_i$ in platelets administered either Me_2SO (*DMSO*; control vehicle) or EIPA (50 μM) 3 min prior to collagen activation. *b*, collagen-induced change in pH$_i$ measured 3 min following the administration of collagen (10 μg/ml) to platelets administered Me_2SO (control vehicle), CBDMB (4 μM), or EIPA (50 μM) 3 min prior to collagen activation. *, $p < 0.05$ ($n = 5$).

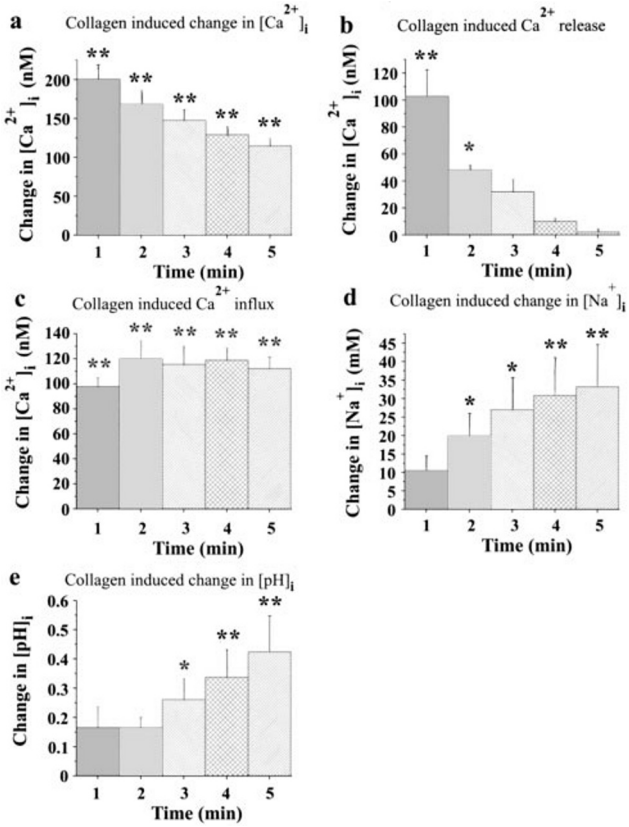

FIG. 7. **Collagen-induced changes in [Ca^{2+}]$_i$, [Na$^+$]$_i$, and [pH]$_i$.** Collagen-induced changes in [Ca^{2+}]$_i$, [Na$^+$]$_i$, and [pH]$_i$ were measured every minute for 5 min following the administration of collagen (10 μg/ml). *a*, total average change in [Ca^{2+}]$_i$. **, $p < 0.005$ ($n = 5$). *b*, change in [Ca^{2+}]$_i$ in platelets suspended in no-Ca^{2+} buffer achieved by the administration of 5 mM EGTA 1 min prior to the administration of collagen, demonstrating the portion of the collagen-induced Ca^{2+} release from intracellular stores. *, $p < 0.05$ ($n = 5$); **, $p < 0.005$ ($n = 5$). *c*, portion of the collagen-induced calcium influx determined by subtracting the amount of calcium released from the intracellular stores (*b*) from the total collagen-induced change in [Ca^{2+}]$_i$ (*a*). **, $p < 0.005$ ($n = 5$). *d*, average collagen-induced change in [Na$^+$]$_i$. *, $p < 0.05$ ($n = 4$); **, $p < 0.005$ ($n = 4$). *e*, average collagen-induced change in [pH]$_i$. *, $p < 0.05$ ($n = 5$); **, $p < 0.005$ ($n = 5$).

crease in the collagen-induced increase in [Ca^{2+}]$_i$ of 87.8 ± 24.8 nM ($n = 5$; $p < 0.005$), whereas inhibition of the NHE by EIPA had no significant effect (Fig. 5*a*). Inhibition of the NCX also produced a decrease of 88.3 ± 7.7% ($n = 5$; $p < 0.005$) in collagen-induced platelet aggregation, whereas inhibition of the NHE by EIPA had no significant effect (Fig. 5*b*). The NCX inhibitors CBDMB and KB-R7943 decreased the collagen-induced changes in [Ca^{2+}]$_i$ in a dose-dependent manner (12). The KD_{50} values measured 30 s following collagen activation were 4.7 ± 1.1 μM for CBDMB ($n = 4$) (Fig. 5*c*) and 35.1 ± 4.8 μM for KB-R7943 ($n = 5$) (Fig. 5*d*).

Collagen-induced platelet activation was associated with an increase in pH$_i$ of 0.26 ± 0.7 ($n = 5$; $p < 0.05$) (Fig. 6*b*) from a base line of 7.47 ± 0.2 ($n = 15$). Pretreatment with the NCX blocker CBDMB (4 μM) did not significantly alter the collagen-induced change in pH$_i$ (Fig. 6*b*). However, inhibition of the NHE by EIPA (50 μM) abolished the collagen-induced increase in pH$_i$ and indeed decreased the pH$_i$ by 0.38 ± 0.11 ($n = 5$; $p < 0.05$) (Fig. 6*b*) compared with the control. Collagen-induced activation of aspirin-pretreated platelets increased the pH$_i$ by 0.10 ± 0.04 ($n = 5$), which is not significantly different from non-aspirin-treated platelets. Pretreatment with either CBDMB (4 μM) or EIPA (50 μM) did not significantly alter the collagen-induced change in pH$_i$ (0.23 ± 0.19 ($n = 5$; not significant) and 0.06 ± 0.07 ($n = 5$; not significant), respectively) compared with the control.

Time Course for the Collagen-induced Changes in [Ca^{2+}]$_i$, [Na$^+$]$_i$, and [pH]$_i$—Collagen induced a rapid increase in platelet [Ca^{2+}]$_i$, which gradually decreased over a 5-min period (Fig. 7*a*). Deducting the collagen-induced Ca^{2+} release (Fig. 7*b*) from the total change in [Ca^{2+}]$_i$ (Fig. 7*a*) established which portion of the collagen-induced increase in [Ca^{2+}]$_i$ is due to collagen-induced Ca^{2+} influx (Fig. 7*c*). The collagen-induced release of intracellular Ca^{2+} was significant only in the first phase of platelet activation, whereas the collagen-induced Ca^{2+} influx predominated throughout. Both [Na$^+$]$_i$ (Fig. 7*d*) and [pH]$_i$ (Fig. 7*e*) were found to increase during the 5 min following the administration of collagen; however, the changes in [pH]$_i$ became significant only at latter time points. In addition, the changes in [pH]$_i$ were often transient.

Role of Thromboxane in the Collagen-induced Entry of Sodium and Calcium—Collagen-induced activation of human platelets resulted in an increase in [Na$^+$]$_i$ of 26.9 ± 8.6 mM from a base line of 33.1 ± 6.4 mM. Pretreatment of platelets with aspirin to inhibit the production of TXA reduced the collagen-induced increase in [Na$^+$]$_i$ to 7.6 ± 5.7 mM ($n = 4$; $p < 0.05$) (Fig. 8*b*).

Aspirin pretreatment of platelets reduced the collagen-induced change in [Ca^{2+}]$_i$ from 147.3 ± 13.3 to 55.4 ± 3.7 nM ($n = 5$; $p < 0.0005$) (Fig. 8*c*) (11, 17) and reduced aggregation by 74.6 ± 1.4% ($n = 5$; $p < 0.0005$) (Fig. 8*d*) (11). The collagen-induced change in [Ca^{2+}]$_i$ in aspirin-pretreated platelets was reduced further by the administration of either the NCX blocker CBDMB at 4 μM (36.3 ± 5.1 nM, $n = 5$; $p < 0.005$) or the NHE blocker EIPA at 50 μM (40.3 ± 3.7 nM, $n = 5$; $p < 0.005$) (Fig. 9*a*). The collagen-induced aggregation of aspirin-pretreated platelets was reduced by 33.1 ± 8.6% ($n = 5$; $p < 0.05$) by CBDMB, whereas EIPA did not result in a significant further reduction in collagen-induced aggregation (7.4 ± 11.9%, $n = 5$; not significant) (Fig. 9*b*). Chelation of extracellular

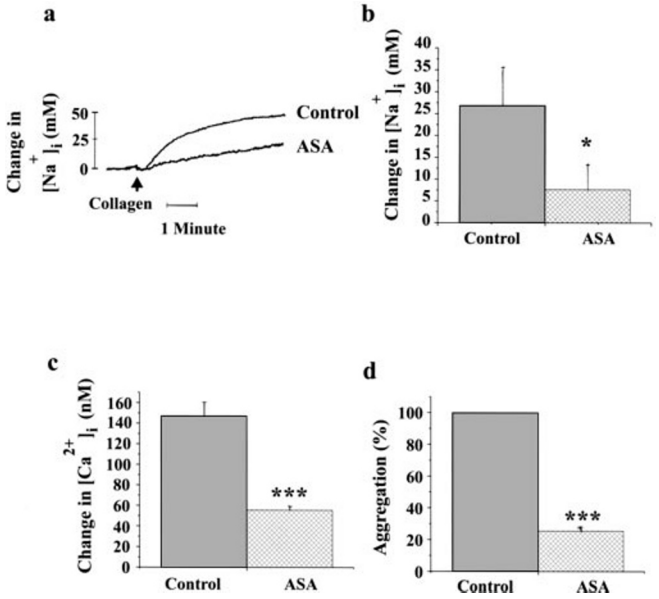

FIG. 8. **Effect of aspirin pretreatment on the collagen-induced changes in $[Ca^{2+}]_i$ and $[Na^+]_i$.** *a*, typical superimposed traces of the collagen-induced change in $[Na^+]_i$ in samples of control and aspirin (*ASA*; 0.5 mM)-pretreated platelets. *b*, collagen-induced change in $[Na^+]_i$ measured 3 min following the administration of collagen (10 μg/ml) to control and aspirin (0.5 mM)-pretreated platelets. *c*, collagen-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of collagen (10 μg/ml) to control or aspirin (0.5 mM)-pretreated platelets. *d*, relative percent change in collagen-induced platelet aggregation measured 2 min following the administration of collagen (10 μg/ml) to platelets pretreated with aspirin (0.5 mM) compared with the control. *, $p < 0.05$ ($n = 4$); ***, $p < 0.0005$ ($n = 5$).

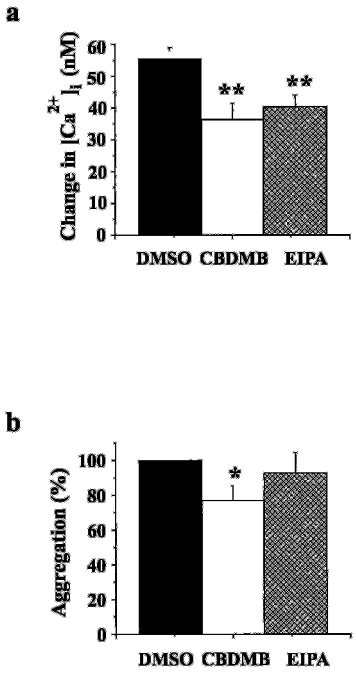

FIG. 9. **Role of the NCX and NHE in collagen activation of aspirin-pretreated human platelets.** *a*, collagen-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of 10 μg/ml collagen to aspirin (0.5 mM)-pretreated platelets suspended in 1 mM Ca^{2+}/HEPES buffer and administered Me_2SO (*DMSO*; control vehicle), 4 μM CBDMB, or 50 μM EIPA 3 min prior to the addition of collagen. *b*, relative percent change in collagen-induced platelet aggregation measured 2 min following the administration of 10 μg/ml collagen to aspirin (0.5 mM)-pretreated platelets suspended in 1 mM Ca^{2+}/HEPES buffer and administered 4 μM CBDMB or 50 μM EIPA 3 min prior to the addition of collagen compared with the control. *, $p < 0.05$ ($n = 5$); **, $p < 0.005$ ($n = 5$).

calcium with EGTA prior to collagen activation of aspirin-pretreated platelets reduced the collagen-induced change in $[Ca^{2+}]_i$ to 9.3 ± 2.2 nM ($n = 5$; $p < 0.0005$) (see Fig. 11*a*).

To further characterize the role TXA plays in platelet activation with collagen, the effects of a stable endoperoxide analog (U-46619) were examined. U-46619 (10 μM) activated human platelets and increased $[Ca^{2+}]_i$ (Fig. 10), which was unaffected by aspirin pretreatment.

We found that ~80% (47.1 ± 2.0 nM, $n = 6$; $p < 0.005$) of the U-46619-induced increase in $[Ca^{2+}]_i$ was due to the influx of calcium from the extracellular milieu (Fig. 11*b*) as judged by chelation of extracellular calcium with EGTA. The U-46619-induced increase in $[Ca^{2+}]_i$ showed a similar sensitivity to sodium compared with collagen. Low-sodium conditions reduced U-46619-induced increases in $[Ca^{2+}]_i$ from a control of 58.9 ± 10.0 to 8.2 ± 5.0 nM ($n = 4$; $p < 0.005$) (Fig. 12*a*) and aggregation by 59.2 ± 10.4% ($n = 4$; $p < 0.005$) (Fig. 12*b*). Similarly, no-sodium buffer reduced the U-46619-induced change in $[Ca^{2+}]_i$ to −4.1 ± 2.1 ($n = 4$; $p < 0.005$) (Fig. 12*a*) and reduced aggregation by 85.0 ± 3.9% ($n = 4$; $p < 0.005$) (Fig. 12*b*).

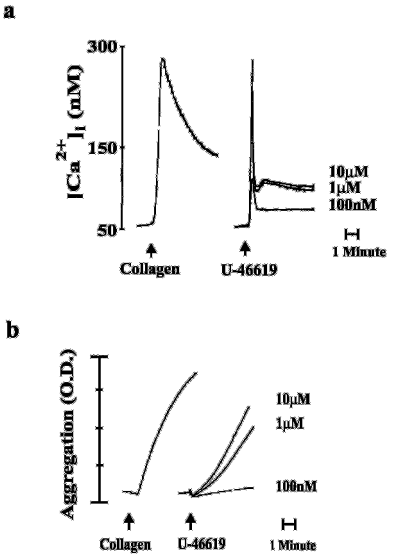

FIG. 10. **Relationship between collagen and U-46619 activation of human platelets.** *a*, typical traces of the change in $[Ca^{2+}]_i$ in platelets suspended in 1 mM Ca^{2+}/HEPES buffer and activated with either collagen (10 μg/ml) or U-46619 (100 nM, 1 μM, or 10 μM). *b*, the corresponding effect on platelet aggregation.

DISCUSSION

Collagen fibers are exposed to circulating platelets following injury to the vessel wall and play an important role in hemostasis through the creation of a physical barrier at the site of vascular damage, thereby limiting blood loss (2–4), and the stimulation of platelet activation, thereby recruiting additional platelets to the site of damage as well as consolidating the thrombus (4). The dual actions of collagen have been explained in the two-step model for activation, which suggests that adhesion and activation of platelets are mediated through the interaction of collagen with platelet integrin $\alpha_2\beta_1$ and glycoprotein VI, respectively (3, 5).

Collagen-induced Platelet Aggregation, a Ca^{2+}-dependent Response—Platelet activation is characterized by shape change, granule secretion, and ultimately, aggregation. These responses are triggered by an increase in $[Ca^{2+}]_i$. Collagen induces a dose-dependent increase in $[Ca^{2+}]_i$ (Ref. 11 and this

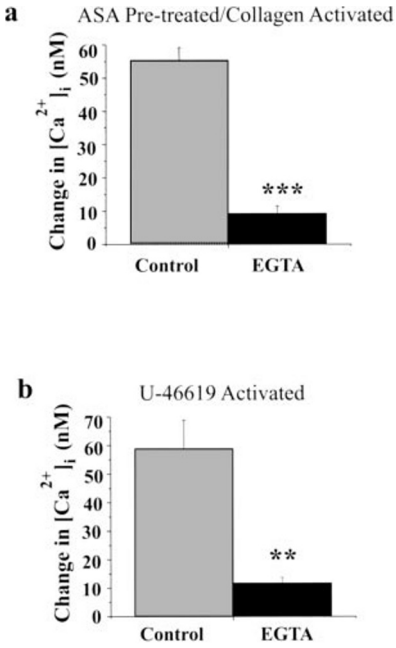

FIG. 11. **Role of extracellular Ca^{2+} in platelet activation.** *a*, collagen-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of 10 μg/ml collagen to aspirin (0.5 mM)-pretreated platelets suspended in 1 mM Ca^{2+}/HEPES buffer (*Control*) or in no-Ca^{2+} medium achieved by the administration of 5 mM EGTA 1 min prior to collagen administration. *b*, U-46619-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of 10 μM U-46619 to platelets suspended in 1 mM Ca^{2+}/HEPES buffer or in no-Ca^{2+} medium achieved by the administration of 5 mM EGTA 1 min prior to the administration of U-46619. **, $p < 0.005$ ($n = 6$); ***, $p < 0.0005$ ($n = 5$).

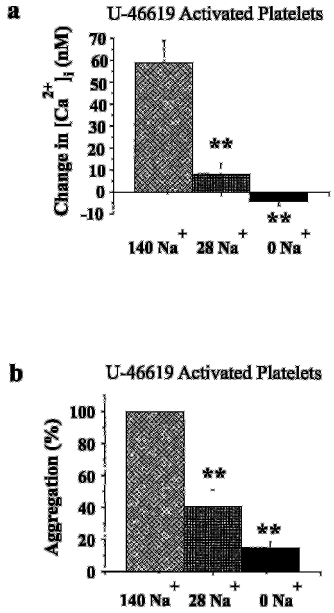

FIG. 12. **Sodium requirement in U-46619 activation of human platelets.** *a*, U-46619-induced change in $[Ca^{2+}]_i$ measured 3 min following the administration of 10 μM U-46619 to platelets suspended in 1 mM Ca^{2+}/HEPES buffer containing 140, 28, or 0 mM Na^+. *b*, relative percent change in U-46619-induced platelet aggregation measured 3 min following the addition of 10 μM U-46619 to platelets suspended in 1 mM Ca^{2+}/HEPES buffer containing either 28 or 0 mM Na^+ compared with the U-46619-induced platelet aggregation of platelets suspended in 1 mM Ca^{2+}/HEPES buffer containing 140 mM Na^+. **, $p < 0.005$ ($n = 4$).

study). Inhibition of this increase in $[Ca^{2+}]_i$ obstructs the collagen-induced aggregation of human platelets. Therefore, an understanding of the mechanisms involved in calcium mobilization will further the understanding of the processes whereby collagen induces platelet aggregation.

Collagen-induced Increase in $[Ca^{2+}]_i$ via the Reverse Mode NCX—Previous studies have demonstrated that the majority of the increase in $[Ca^{2+}]_i$ in response to a moderate dose of collagen (10 μg/ml) is due to the influx of calcium from the extracellular milieu (12, 40). Platelet membranes contain the NCX (20, 21), and it has been shown that the mode of action of the NCX can be reversed, leading to an increase in $[Ca^{2+}]_i$ (20, 21, 42). This study has shown that the collagen-induced increase in $[Ca^{2+}]_i$ is dependent on the concentration gradient of Na^+ and that blockage of the NCX reduces the collagen-induced increase in $[Ca^{2+}]_i$. Taken together, these data suggest that the Ca^{2+} influx in response to collagen is due to the actions of the NCX functioning in the reverse mode and thus differs from thrombin activation (34).

Calcium influx through the NCX is dependent on the sodium gradient. An increase in $[Ca^{2+}]_i$ when external Na^+ is removed is consistent with the presence of the NCX functioning in the reverse mode (20, 21, 42, 43). Reducing the sodium gradient had no effect on resting platelet $[Ca^{2+}]_i$, indicating that, in non-activated platelets, the NCX is not functioning in a reverse mode. However, collagen-induced activation of the platelets in a low- or no-sodium medium resulted in a dose-dependent decrease in the collagen-induced increase in $[Ca^{2+}]_i$, consistent with the increase in $[Ca^{2+}]_i$ being dependent on the sodium gradient and therefore resulting from the reversal of the NCX. This is in contrast to the effect of low sodium on thrombin, where an increase in $[Ca^{2+}]_i$ is observed due to inhibition of the NCX operating in the forward mode (40).

The necessity of extracellular Na^+ in activation of human platelets is not unique to collagen. Epinephrine, ADP, and low-dose thrombin demonstrate reduced platelet activation in the absence of external Na^+ (44, 45). To determine whether this is related to the absence of Na^+ as opposed to an alteration in the osmolarity or ionic composition of the buffer, Na^+ was replaced with iso-osmolar sucrose or with equimolar *N*-methylglucamine or choline chloride. Under these conditions, a significant reduction in the collagen-induced change in $[Ca^{2+}]_i$ was observed with the substitution of Na^+, confirming previous observations (12). Thus, it can be concluded that the effects we have observed are not due to osmolar or ionic changes, but rather to the absence of external Na^+.

Although the intracellular release of Ca^{2+} in response to collagen is small in comparison with the extracellular influx, its contribution cannot be discounted. Chelation of intracellular calcium by BAPTA did not result in a significant reduction in the collagen-induced increase in $[Ca^{2+}]_i$; however, it did cause a significant reduction in collagen-induced aggregation. These observations may be the result of the actions of localized immeasurable changes in the $[Ca^{2+}]_i$, or it is possible that the collagen-induced influx of extracellular Ca^{2+} saturated BAPTA in a way that it was unable to give an accurate reflection of the collagen-induced release of intracellular calcium. Combining intracellular calcium chelation with a decrease in extracellular sodium caused a significant decrease in the collagen-induced increase in $[Ca^{2+}]_i$ and almost completely abolished aggregation. In contrast, the potent protein kinase C agonist phorbol 12,13-dibutyrate (1 μM) initiated platelet aggregation that was unaffected by the chelation of intracellular calcium in the presence or absence of extracellular sodium (data not shown).

The collagen-induced influx of Ca^{2+} through the reverse mode NCX is further substantiated by the reduction in both $[Ca^{2+}]_i$ and aggregation through the administration of the NCX blocker CBDMB. CBDMB and KB-R7943, a reverse mode-spe-

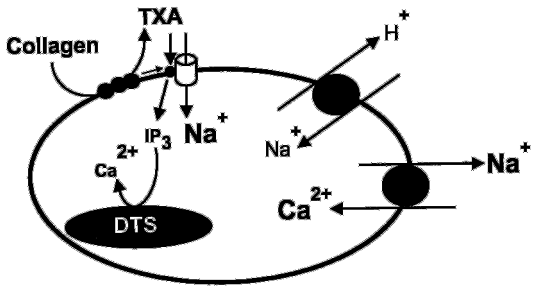

FIG. 13. **Collagen-induced calcium mobilization.** The proposed mechanism for the collagen-induced calcium mobilization in human platelets is shown. Collagen induces the production of TXA and subsequently inositol 1,4,5-trisphosphate (*IP_3*), which mobilizes calcium from the dense tubular system (*DTS*). TXA and, to a lesser extent, the NHE cause the influx of Na^+ into the platelet cytosol. The increase in $[Na^+]_i$ causes the reversal of the NCX and a further increase in $[Ca^{2+}]_i$.

cific NCX blocker (46), demonstrated a dose-dependent inhibition of the collagen-induced change in $[Ca^{2+}]_i$.

Collagen-induced Increase in $[Na^+]_i$—The NCX mode of action is dependent on the Na^+ gradient (20, 21, 42, 43), and several studies have shown that the NCX reversal occurs following an increase in $[Na^+]_i$ (21, 42). Therefore, it seems likely that the collagen-induced reversal of NCX function must be a result of the disruption in the platelet Na^+ gradient. Because collagen-induced increases in $[Ca^{2+}]_i$ occur when external Na^+ is maintained at 140 mM, it can then be reasoned that NCX reversal is achieved through a collagen-induced increase in $[Na^+]_i$. Agonist-induced Na^+ influx has been observed in response to the platelet activators thrombin and ADP (29, 30) and collagen (as shown here). In the case of thrombin and ADP, part of the Na^+ influx has been attributed to activation of the NHE; however, inhibiting the NHE has no effect on the collagen-induced increase in $[Ca^{2+}]_i$ or aggregation, suggesting another mechanism for the collagen-induced increase in $[Na^+]_i$.

The NHE mediates the exchange of extracellular Na^+ for intracellular H^+, thereby regulating pH_i. An increase in pH_i subsequent to platelet activation enhances calcium mobilization in response to various platelet activators (9, 20, 47). The present results demonstrate that collagen activation of platelets results in an increase in pH_i. Previous studies by Rengasamy *et al.* (20) demonstrated that Na^+-dependent Ca^{2+} uptake is enhanced at high pH_i. Therefore, collagen alkalization of the platelet cytosol may be another mechanism that can further increase $[Ca^{2+}]_i$ during platelet activation.

Collagen-induced Production of TXA Enhances the Increase in $[Ca^{2+}]_i$ and $[Na^+]_i$—TXA, produced subsequent to platelet activation with collagen (48), enhances collagen-induced activation (6, 13–15). Inhibiting TXA production with aspirin reduced the collagen-induced increase in $[Ca^{2+}]_i$ and aggregation and was almost completely abolished by the chelation of extracellular Ca^{2+}. Furthermore, pretreating platelets with aspirin drastically decreased the collagen-induced increase in $[Na^+]_i$, suggesting that TXA enhances collagen activation of platelets through both mobilization of Ca^{2+} and influx of Na^+ with associated disruption of the Na^+ gradient.

The Ca^{2+} influx following collagen activation of aspirin-pretreated platelets was also through the actions of the NCX functioning in the reverse mode as judged by the affects of NCX blockers. Furthermore, blockage of the NHE in aspirin-pretreated platelets resulted in a reduction in the collagen-induced change in $[Ca^{2+}]_i$, indicating that the NHE plays a role in disrupting the Na^+ gradient required for the reversal of the NCX. We believe that the actions of the NHE are not limited to aspirin-pretreated platelets, but rather are masked by the actions of TXA. The role of the NHE in platelet activation becomes apparent only when TXA production is inhibited.

These results indicate a novel role for the NCX in platelet activation. It functions in the forward mode to maintain calcium homeostasis; and following the administration of collagen, it adopts a key role as an initiator of platelet activation. This role is made possible by the influx of sodium via the actions of TXA and the NHE as summarized in Fig. 13.

Conclusion—The experimental evidence presented here indicates that collagen activation of human platelets requires an increase in their $[Ca^{2+}]_i$, the majority of which is due to the influx of Ca^{2+} through the NCX functioning in a reverse mode. NCX reversal occurs subsequent to the influx of Na^+ due to the actions of TXA, and to a lesser extent by the NHE. TXA thus amplifies the actions of collagen.

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