Cyclic nucleotides attenuate thrombin-evoked alterations in parameters of platelet Na/H antiport. The role of cytosolic Ca.

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Research Article

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Cyclic Nucleotides Attenuate Thrombin-evoked Alterations in Parameters of Platelet Na/H Antiport

The Role of Cytosolic Ca

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Abstract

In this work, we explored the role of cyclic nucleotides in modulating parameters of the Na/H antiport in human platelets. Sodium nitroprusside and iloprost, as well as cyclic nucleotide analogues, were used to raise cellular levels of cAMP and cGMP. Cyclic nucleotides reversed the thrombin-evoked alkaline shift in cytosolic pH set point and the activity of the Na/H antiport, concurrently with attenuation of thrombin-induced rise in cytosolic free Ca. No effect of cyclic nucleotides was observed in platelets not treated with thrombin, or platelets subjected to phorbol 12-myristate 13-acetate. cAMP did not reverse ionomycin-induced changes in the parameters of the Na/H antiport. Collectively, these observations indicate that cyclic nucleotides modulate the Na/H antiporter in human platelets through their effect on thrombin-evoked changes in cytosolic free Ca. Presumably, this effect holds for other agonists which stimulate phospholipase C, raise cytosolic-free Ca, and activate the Na/H antiport through protein kinase C dependent and protein kinase C-independent mechanisms. (*J. Clin. Invest.* 1992. 89:1121–1127.) Key words: cyclic AMP • cyclic GMP • cytosolic pH • protein kinase C • phorbol esters

Introduction

Activation of platelets by a variety of agonists is associated with stimulation of the Na/H antiport (1–3). This mode of activation of the Na/H antiport usually entails stimulation of phospholipase C (PLC),[1] a rise in cytosolic-free Ca (Ca_i), and stimulation of protein kinase C (PKC) (3–5; for review, see reference 6). However, it can proceed through PKC-independent mechanisms. The Na/H antiport can also be activated without an apparent rise in Ca_i, or stimulation of PLC. For instance, phorbol esters circumvent PLC by activating the Na/H antiport through direct stimulation of PKC (2, 7–10). Irrespective of the pathway of agonist-evoked stimulation of the Na/H antiport, this process is accomplished by an alkaline shift in the cytosolic pH (pH_i) set point for activation, by increased activity of the Na/H antiport within a given pH range, or by both mechanisms (2, 6).

In a previous study, we showed that Ca_i is central to agonist and nonagonist-mediated activation of the Na/H antiport in human platelets, and that the effect of Ca_i predominates over that of PKC (2). Cyclic nucleotides are cellular messengers that inhibit platelet aggregation and diametrically antagonize Ca_i in these cells (11–16). We have thus focused this investigation on the role of cyclic nucleotides in modulating parameters of the Na/H antiport. Our results indicate that cyclic nucleotides exert their effect on the Na/H antiport primarily through Ca_i.

Methods

Platelet preparation

50 ml of blood were drawn from each subject into acid dextrose buffer (20:1, vol/vol) consisting of (mM) sodium citrate 14, citric acid 11.8, and dextrose 18 (final pH 6.5). Platelet-rich plasma was obtained by centrifugation at 200 *g* for 10 min at room temperature. It was then centrifuged at 1,000 *g* for 10 min, and cells were washed three times (using centrifugations at 1,000 *g*) with buffer consisting of (mM) NaCl 140, KCl 5, glucose 10, EGTA 0.2, Hepes 10, and (unless otherwise indicated) acetylsalicylic acid (aspirin) 0.1. BSA (0.1%) was added to the third washing, and EGTA was deleted. BSA was included in all subsequently used solutions, except the dye loading buffer. Aspirin was used in most platelet preparations because (*a*) Ca_i and pH_i signals under basal and stimulated states were more stable in aspirin-treated platelets; and (*b*) stimulation of the Na/H antiport may promote agonist-induced arachidonic acid release (1) that, in turn, can accelerate Ca influx and mobilization by cyclooxygenase metabolites (17). Such a process would render it difficult to assess the effect of Ca_i on parameters of the Na/H antiport, without the confounding effect of a positive feedback of the Na/H antiport on Ca_i. Platelets were incubated respectively with 5 μM 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetomethyl ester (BCECF-AM), or fura 2 acetometyl ester (fura 2-AM) (Molecular Probes, Inc., Eugene, OR) for 60 and 30 min at 37°C in Hepes-buffered solution (HBS) consisting of (mM) NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, and Hepes 10 (pH 7.4).

Aliquots (100 μl) of platelets loaded with either BCECF or fura-2 were centrifuged at room temperature for 7s (maximal centrifugation force at 7s = 3,000 *g*). Thereafter, some platelets underwent measurements of basal pH_i and Ca_i in HBS (pH 7.4). These nonacidified platelets were also subjected to thrombin or other agents, including dibutyryl cyclic AMP (Bt_2cAMP), 8 bromo cyclic GMP (8BrcGMP), sodium nitroprusside (SNP), iloprost (Berlex Laboratories, Wayne, NJ), ionomycin, phorbol 12-myristate 13-acetate (PMA), and cAMP-de-

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1. *Abbreviations used in this paper:* Bt_2cAMP, dibutyryl cAMP; Ca_i, cytosolic-free Ca; 8BrcGMP, 8 bromo cGMP; HBS, HEPES-buffered solution; pH_i, cytosolic pH; PLC, phospholipase C; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SNP, sodium nitroprusside; Xi, the set point for activation of the Na/H antiport; Zi, activity index.

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pendent protein kinase inhibitor KT5720 (Kamiya Biochemical Corp., Thousand Oaks, CA). Other platelets were treated with the various agonists and probes before and during cellular acidification and other experimental manipulations. For cellular acidification, 80 μl of dye-loaded platelets in Na-free HBS (*N*-methyl-D-glucamine replacing Na) were injected into cuvettes containing 3 ml HBS (in which 20–140 mM Na propionate equimolarly replaced NaCl; pH 7.4), with or without thrombin and other agents. Whenever indicated, platelets were preincubated in HBS containing these agents before their injections into the cuvette, which also contained these agents in HBS. Cellular buffering power was determined as previously described (2).

pH_i and Ca_i monitoring. Monitoring of cellular fluorescence was performed at 37°C under constant stirring in a Flurolog II spectrofluorimeter (Flurolog II model CM-3; SPEX Inds. Inc., Edison, NJ). Excitation and emission wavelengths for pH_i measurements were 440/503 and 530 nm, respectively. For Ca_i measurements, the respective values for excitation and emission wavelengths were 340/380 and 505 nm. For further details, see reference 2.

Data analysis. Initial rates for the pH_i recovery from acidification were derived from iterative curve fittings, as described previously (18). These rates represent the activity of the Na/H antiport, inasmuch as the recovery was Na-dependent and sensitive to amiloride analogues. The initial rates demonstrate a linear relationship with the initial pH_i when the latter parameter is expressed as cellular proton concentration. The *x*-axis intersect of the linear regression describing the initial rates of recovery from acidifications as a function of the cellular proton concentration represents the set point, and the slope of the line the activity index (Zi) of the Na/H antiport. When expressed in pH units, the set point for activation of the Na/H antiport is termed Xi (for further details, see reference 2).

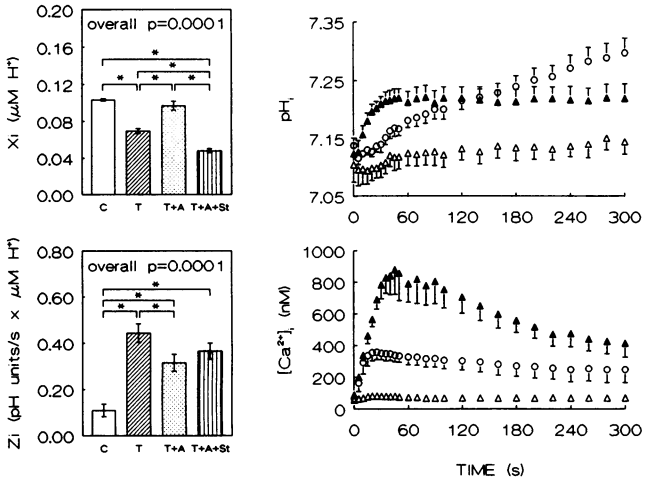

Figure 1. The effect of a combination of Bt_2cAMP (3 mM) and staurosporine (5 μM) on the thrombin-induced response. Left panels summarize the Xi and Zi data. Right panels describe the pH_i and Ca_i profiles in nonacidified platelets. *C*, control platelets; *T*, thrombin treated platelets; *T + A*, thrombin-treated platelets pretreated with 3 mM Bt_2cAMP; T + A + St, thrombin-treated platelets preincubated with Bt_2cAMP; and 5 μM staurosporine. When expressed in pH units, Xi for C = 6.986, for T = 7.159, for T + A = 7.013 and for T + A + St = 7.319. In the right panels, *open circles,* thrombin, *open triangles,* T + A, and *closed triangles,* T + A + St. Thrombin raised the pH_i from 7.138 to 7.298 at 300 s, and the Ca_i from 65.8 to a peak of 358 at 25 s, and to 247 nM at 300 s. In the presence of Bt_2cAMP, thrombin raised the pH_i from 7.105 to 7.145 at 300 s, and the Ca_i from 56.4 to a maximum of 82.2 at 20 s, and to 68.3 nM at 300 s. In platelets pretreated with both Bt_2cAMP and staurosporine, thrombin raised the pH_i from 7.124 to 7.220 at 300 s, and the Ca_i from 89 to a maximum of 880 at 45 s, and 413 nM at 300 s (number of subjects = 3).

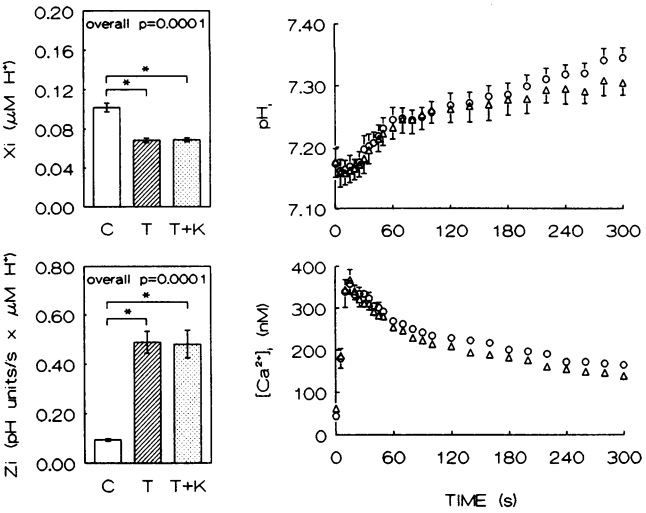

Figure 2. The effect of KT5720 (1 μM) on the thrombin-induced platelet response. Left panels summarize the Xi and Zi data. Right panels describe the pH_i and Ca_i profiles in nonacidified platelets. *C*, control; *T*, thrombin pretreated platelets; *T + K*, thrombin treated platelets, pretreated with 1 μM of KT5720. When expressed in pH units, Xi for C = 6.992; for T = 7.166 and for T + K = 7.162. In the right panels, *open circles,* thrombin alone; *open triangles,* T + K.

One-way analysis of variance (ANOVA) was used in data analysis. All computations were performed with an IBM compatible PC (SAS REG and GLM programs; SAS Institute, Cary, NC). Data are presented as mean or mean±SEM. Unless indicated, platelets from at least four subjects were used for each experimental protocol.

Results

No statistically significant differences were observed in pH_i and Ca_i under basal or stimulated states between aspirin-treated platelets and platelets not treated with aspirin. For instance, the basal pH_i for platelets not treated with aspirin was 7.097±0.014 (not shown). The basal pH_i levels of aspirin-treated platelets used in experimental protocol described in Figs. 1–4, were 7.138±0.013, 7.099±0.023, 7.123±0.012, and 7.096±0.028, respectively. The effects of the various treatments on Xi or Zi were also equivalent in aspirin-treated platelets and platelets not treated with aspirin. Thus, all further experiments were performed in aspirin-treated platelets.

In general, treatment with thrombin produced transient acidification, followed by alkalinization, and a Ca_i transient that was followed by sustained elevation (compared with basal level) of the posttransient Ca_i.

Fig. 1 summarizes experiments designed to explore the effect of Bt_2cAMP on the thrombin-evoked responses, and the potential effect of PKC inhibition by staurosporine on this process. In itself, thrombin produced an alkaline shift in the Xi, an increase in Zi, and an increase in the Ca_i. The thrombin-evoked alkaline shift in the Xi was reversed, and the thrombin-induced rise in Zi attenuated by treatment with Bt_2cAMP. In nonacidified platelets, the alkalinization produced by thrombin was also reversed by Bt_2cAMP. Likewise, the thrombin-induced Ca_i transient and posttransient Ca_i were practically abolished by the cyclic nucleotide. Preincubation with a combination of Bt_2cAMP staurosporine produced a further alkaline

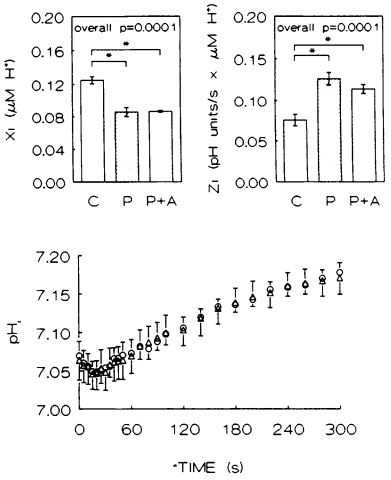

Figure 3. The effect of Bt_2cAMP on PMA-induced alterations of pH_i parameters. Upper panels summarize the Xi and Zi data. *C*, control platelets; *P*, PMA (100 nM)-treated platelets; *P + A*, platelets treated with PMA and 3 mM Bt_2cAMP. Platelets were preincubated for 5 min in the presence of both PMA and Bt_2cAMP before initiation of experiments to determine the Xi and Zi. When expressed in pH units, the Xi for C = 6.903, for P = 7.063, and for P + A = 7.062. Bottom panel describes the pH_i profile in nonacidified platelets. PMA (*open circles*) was added at time = 0. In other experiments, platelets were pretreated for 5 min with Bt_2cAMP before the addition of PMA (*open triangles*). PMA raised the pH_i from 7.070 to 7.178 at 300 s. In the presence of Bt_2cAMP, PMA raised the pH_i from 7.064 to 7.171 at 300 s.

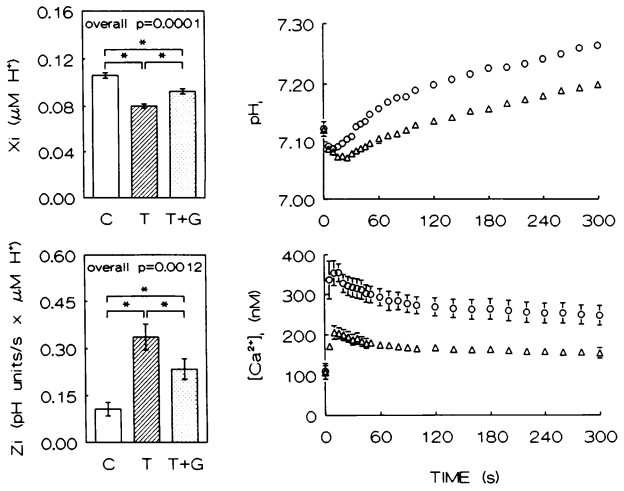

Figure 5. The effect of 8BrcGMP on thrombin-evoked alterations in pH_i and Ca_i parameters. Left panels summarize the Xi and Zi data. *C*, control platelets; *T*, thrombin (0.1 NIH units/ml)-treated platelets; *T + G*, platelets subjected to 5 mM 8BrcGMP for 5 min, and subsequently to thrombin plus 8BrcGMP. When expressed in pH units, Xi for C = 6.974, for T = 7.096, and for T + G = 7.032. Right panels show the thrombin-evoked pH_i and Ca_i profiles in nonacidified platelets. *Open circles*, thrombin-treated platelets; *open triangles*, platelets subjected to thrombin and 8BrcGMP. Thrombin increased pH_i from basal level of 7.122 to 7.264 at 300 s, and Ca_i from basal 110 nM to a maximum of 355 nM at 15 s, and to 248 nM at 300 s. Platelets subjected to both thrombin and 8BrcGMP demonstrated a rise of pH from basal 7.120 to maximum of 7.199 at 300 s, and from basal Ca_i of 107 nM to a maximum of 206 nM at 10 s, and to 155 nM at 300 s.

shift in the Xi than thrombin alone (control = 6.986; thrombin = 7.159; thrombin plus Bt_2cAMP = 7.013; thrombin plus Bt_2cAMP plus staur = 7.319). Moreover, this greater shift in the Xi was coupled with a greater thrombin-induced rise in Ca_i.

Staurosporine can inhibit not only PKC, but also cAMP-dependent protein kinase. To rule out the possibility that the effect of staurosporine is exerted through inhibition of cAMP-dependent kinase, platelets were preincubated for 5 min with 1 μM of KT5720, which is an inhibitor of the enzyme. At this concentration, there is little effect of KT5720 on PKC, yet its K_i for cAMP-dependent protein kinase is $\sim$ 0.05 μM (19, 20). There was no apparent effect of KT5720 on the thrombin-induced cellular response (Fig. 2).

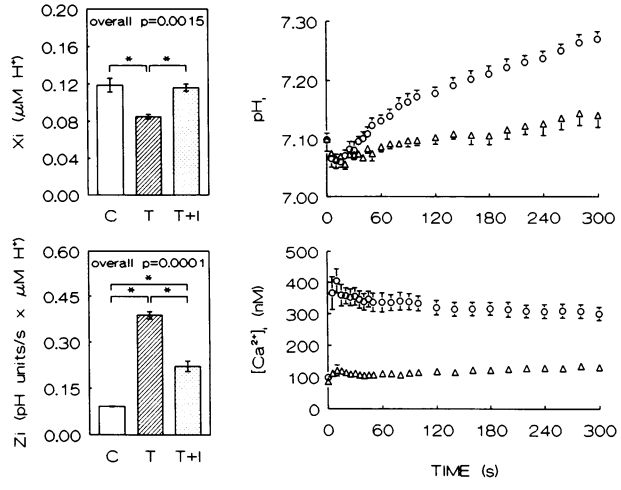

Figure 4. The effect of iloprost on thrombin-evoked alterations in pH_i and Ca_i parameters. In the left panels *C*, control platelets; *T*, thrombin (0.1 NIH units/ml)-treated platelets; *T + I*, platelets subjected to 10 nM iloprost for 5 min, and then subjected to thrombin and iloprost. When expressed in pH units, Xi for C = 6.926, for T = 7.070, and for T + I = 6.935. Right panels show thrombin-evoked changes in pH_i and Ca_i of nonacidified platelets subjected to thrombin alone, *open circles;* and platelets treated with both thrombin and iloprost, *open triangles.* Thrombin was added at time = 0. It caused a rise of pH_i from basal level of 7.099 to 7.270 (300 s), and a rise in Ca_i from basal level of 98.5 nM to a maximum of 405 nM (10 s), and to 300 nM at 300 s. Iloprost treatment resulted in a thrombin-induced rise in pH_i from basal level of 7.100 to 7.141 at 300 s, and from basal Ca_i of 86.2 nM to a maximal level of 131 nM at 300 s.

To further examine the relationship between cyclic nucleotides and PKC, platelets were treated with the phorbol ester PMA and Bt_2cAMP. As shown in Fig. 3, 100 nM of PMA produced an alkaline shift in the Xi from 6.903 to 7.066, and a 67% increase in the Zi. These alterations were not reversed by 3 mM Bt_2cAMP (Xi in the presence of Bt_2cAMP and PMA = 7.062). There was no effect of PMA on Ca_i (not shown). It is noteworthy that in this set of experiments, PMA, like thrombin, induced a transient cellular acidification followed by sustained alkalinization (Fig. 3, *bottom*).

Iloprost is a stable prostacyclin analogue, and it promotes inhibition of platelet aggregation by raising endogenous cAMP (21–23). It was thus used to examine the effect of cAMP on thrombin-evoked alteration in platelet Na/H antiport parameters. The effect of 10 nM iloprost was the same as that of Bt_2cAMP, i.e., reversing the thrombin-induced alkaline shift in Xi (control = 6.926; thrombin = 7.070; thrombin plus iloprost = 6.935) and attenuating the increase in Zi (Fig. 4, *left*). These effects were accompanied by substantial attenuations of the pH_i (alkalinization) and Ca_i responses to thrombin (Fig. 4, *right*).

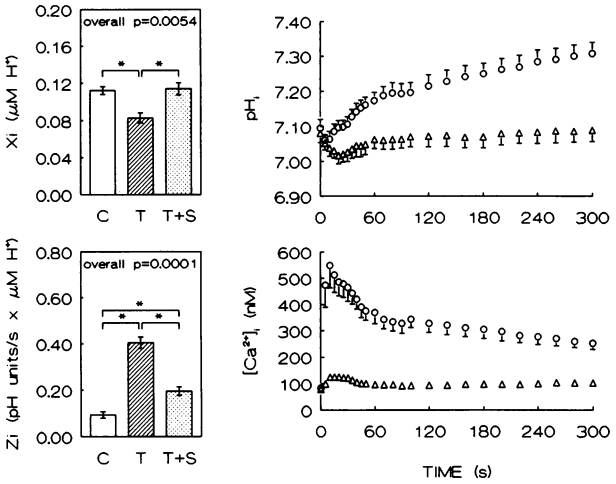

Figure 6. The effect of SNP on thrombin-evoked alterations in pH_i and Ca_i parameters. Left panels summarize the Xi and Zi data. *C*, control platelets; *T*, thrombin (0.1 NIH units/ml)-treated platelet; *T + S* = platelets pretreated for 5 min with 5 mM SNP, and subsequently subjected to both thrombin and SNP. Expressed in pH units, the Xi for C = 6.951, for T = 7.083, and for T + S = 6.943. Right panels show the thrombin-evoked alterations in pH_i and Ca_i in nonacidified platelets. *Open circles,* thrombin-treated platelets and *open triangles,* platelets treated with SNP and thrombin. Thrombin was added at time = 0. Thrombin raised the pH_i from basal level of 7.096 to 7.308 at 300 s, and the Ca_i from 83.1 nM to a maximum of 549 nM at 10 s, and to 253 nM at 300 s. In platelets subjected to SNP, thrombin raised the pH_i from basal level of 7.080 to 7.089 at 300 s, and the Ca_i from 78.1 nM to a maximum of 125 nM at 10 s, and to 103 nM at 300 s.

Thrombin-induced alkaline shift of Xi and increase in Zi of the Na/H antiport were also attenuated by 5 mM 8BrcGMP (Fig. 5) and 5 mM SNP (Fig. 6). The latter compound stimulates guanylate cyclase to raise the endogenous levels of platelet cGMP and inhibits platelet aggregation (11, 12, 24, 25). These effects of endogenous and exogenous cGMP were coupled with marked inhibitions of the Ca_i and pH_i responses to thrombin (Figs. 5 and 6, *right*).

Both Bt_2cAMP (Fig. 7) and SNP (Fig. 8) also reversed thrombin-evoked changes in Xi and inhibited the pH_i and Ca_i responses when experiments were performed in Ca-free HBS ($CaCl_2$ was deleted and 0.3 mM EGTA added). Under these conditions, basal Ca_i and/or the thrombin-evoked transient and posttransient Ca_i levels were, by and large, lower than in 1 mM Ca HBS. It should be noted, however, that in Ca-free HBS only SNP reversed the thrombin effect on the Zi. Bt_2cAMP reduced the Zi of thrombin-treated platelets toward basal level, but the reduction was not statistically significant. There were no effects of Bt_2cAMP and SNP on Xi and Zi (not shown), basal Ca_i, and pH_i (legends to figures) in platelets not treated with thrombin.

Nonagonist-mediated elevation of Ca_i (produced by 500 nM ionomycin in 1 mM HBS) caused an alkaline shift in the Xi from 6.908 to 7.263, and a threefold increase in the Zi (Fig. 9, *left*). Bt_2cAMP neither reversed the ionomycin-induced alkaline shift in Xi (in the presence of Bt_2cAMP and ionomycin, Xi = 7.239) nor attenuated the increase in Zi. Moreover, there was no effect of the cyclic nucleotide on ionomycin-evoked changes in the pH_i and Ca_i profiles in nonacidified platelets (Fig. 9, *right*).

Finally, there was no effect of the various probes on the cellular buffering power (control = 23.9±0.41, Bt_2cAMP = 24.0±0.46, 8BrcGMP = 23.9±0.81, Iloprost = 23.7±0.63, and SNP = 23.5±0.42 mmol × l^{-1} × pH^{-1}).

Discussion

Cyclic nucleotides, i.e., cAMP and cGMP, inhibit agonist-evoked platelet aggregations (12, 23, 26). This inhibition is mediated through lowering the Ca_i (11–16, 27) and via Ca-independent mechanisms (28). Blunting or ablating of agonist-evoked Ca_i response by cyclic nucleotides involve key elements of Ca_i homeostasis, including inhibition of (*a*) agonist-receptor interaction (29); (*b*) PLC (11, 12, 30, 31); (*c*) Ca mobilization from intracellular organelles (26, 27, 30, 32); and (*d*) Ca uptake from the extracellular compartment (27, 30, 32). Additionally, cyclic nucleotides stimulate Ca sequestration (33). Irrespective of the specific mechanisms by which cyclic nucleotides lower the Ca_i, cAMP and cGMP do not lower Ca_i in platelets under basal conditions, or when the Ca_i is raised by Ca ionophores, as has been demonstrated here by us and by others (26, 28, 30, 34).

The central conclusion emerging from the present work is that the effects of cAMP and cGMP on parameters of the Na/H antiport are exerted through attenuation of the Ca_i response to

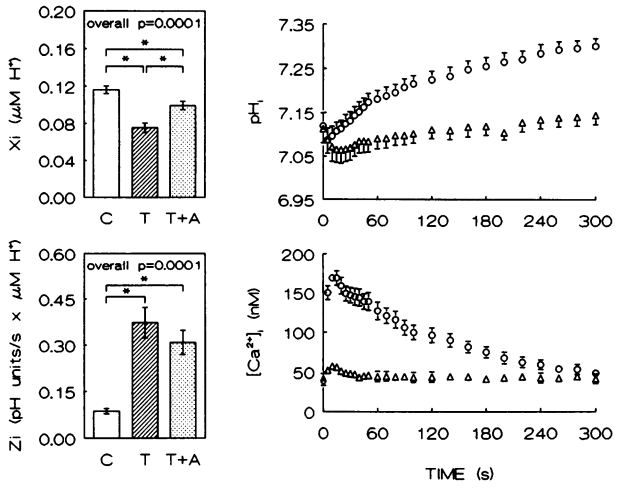

Figure 7. The effect of Bt_2cAMP on thrombin-induced changes in pH_i and Ca_i parameters in Ca-free HBS. Control and experimental platelets were preincubated for 5 min with or without Bt_2cAMP in Ca-free medium. Left panels summarize the Xi and Zi data. *C*, control platelets; *T*, thrombin (0.1 NIH units/ml)-treated platelets; *T + A*, platelets subjected to 3 mM Bt_2cAMP for 5 min, and then to thrombin plus Bt_2cAMP. When expressed in pH units, the Xi for C = 6.934, for T = 7.105, and for T + A = 7.002. Panels on the right depict the pH_i and Ca_i responses to thrombin (added at time = 0) in nonacidified platelets. Thrombin-treated platelets, *open circles;* platelets treated with Bt_2cAMP and thrombin, *open triangles.* Thrombin raised the pH_i from basal level of 7.120 to 7.302 at 300 s, and the Ca_i from 38.2 nM to a maximum of 163.9 nM at 10 s, and 48.7 nM at 300 s. In platelets treated with thrombin and Bt_2cAMP, thrombin raised the pH_i from 7.113 to 7.146 at 300 s, and from a basal Ca_i of 42.4 nM to a maximum of 57.3 nM at 10 s, and 41.7 nM at 300 s.

thrombin. These agents had no effect on the Xi, Zi, and pH_i profile under circumstances in which they did not modulate the Ca_i (e.g., basal conditions, treatment with ionomycin, treatment with PMA). Moreover, the Xi manifests greater sensitivity to cyclic nucleotides than the Zi. In previous work, we suggested that the Xi and Zi demonstrate different dependencies on Ca_i (2). It is thus possible that the Na/H antiport exhibits more than one mode of operation at various Ca_i levels, or that more than one isomer of this transport system exists in human platelets.

The inability of cyclic nucleotides to completely block the thrombin-evoked rise in the Zi appears unrelated to thrombin-induced stimulation of PKC. Inhibition of PKC, in fact, produced a further alkaline shift in the Xi in thrombin and Bt_2cAMP-treated platelets. This is likely to relate to the further rise in the Ca_i in response to thrombin in platelets treated with staurosporine (for further details, see reference 2). It is possible, however, that because of its nonspecificity, staurosporine not only inhibited PKC but also cAMP-dependent protein kinase. Thus, treatment with staurosporine could blunt cAMP action if it is mediated through cAMP-dependent protein kinase. The use of the specific inhibitor of this enzyme (KT5720) showed that this was not the case.

Inhibition of the Na/H antiport by cAMP has been demonstrated in other cells, such as epithelia of the urinary system (35–38), and in osteoblast-like cells (39). Thus, agents known

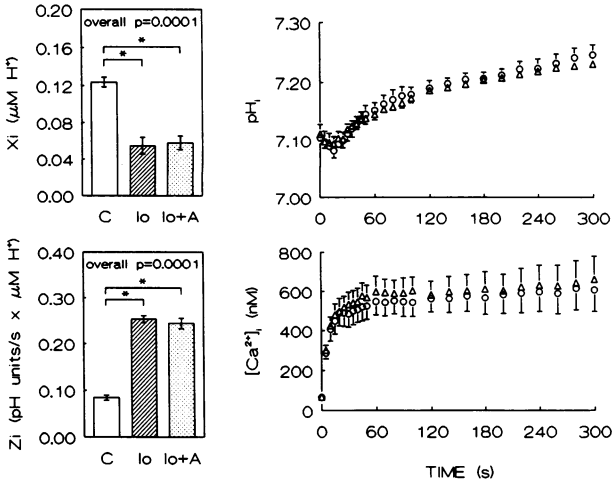

Figure 9. The effect of Bt_2cAMP on ionomycin-induced alterations in pH_i and Ca_i parameters. Left panels summarize the Xi and Zi data. *C,* control platelets; *Io,* ionomycin (500 nM)-treated platelets; *Io + A,* platelets preincubated with a combination of 3 mM Bt_2cAMP and ionomycin. When expressed in pH units, Xi for C = 6.908, for Io = 7.263 and for Io + A = 7.239. Right panels describe the pH_i and Ca_i profiles in nonacidified platelets. *Open circles,* ionomycin-treated platelets; *open triangles,* platelets treated with Bt_2cAMP and subsequently with ionomycin. Ionomycin raised the pH_i from 7.104 to 7.246 at 300 s, and the Ca_i from 65.2 nM to 608 nM at 300 s. In Bt_2cAMP-treated platelets, ionomycin raised the pH_i from 7.111 to 7.231 at 300 s, and the Ca_i from 67.3 nM to 663 nM, at 300 s.

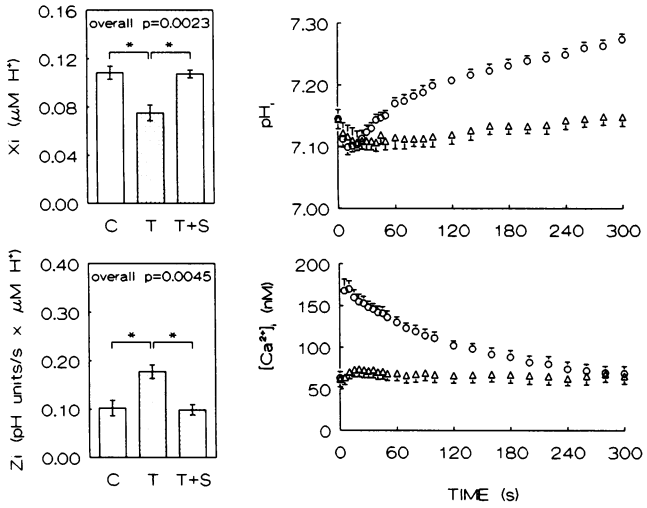

Figure 8. The effect of SNP on thrombin-induced alterations in pH_i and Ca_i parameters in Ca-free HBS. Control and experimental platelets were preincubated for 5 min in Ca-free medium with or without SNP. Left panels summarize the Xi and Zi data. *C,* control platelets; *T,* thrombin (0.1 NIH units/ml)-treated platelets; *T + S,* platelets treated for 5 min with 5 mM SNP, and subsequently subjected to both thrombin and SNP. When expressed in pH units, Xi for C = 6.965, for T = 7.125, and for T + S = 6.969. Panels on the right describe the pH_i and Ca_i profiles in response to thrombin in nonacidified platelets. Thrombin-treated platelet, *open circles;* platelets treated with SNP and thrombin, *open triangles.* Thrombin increased the pH_i from 7.145 to 7.274 at 300 s, and the Ca_i from 63.1 nM to a maximum of 170 nM at 10 s, and to 68.6 nM at 300 s. In SNP-treated platelets, thrombin raised the pH_i from 7.145 to 7.148 at 300 s, and the Ca_i from 61.7 nM to a maximum of 73.8 nM at 20 s, and to 65.5 nM at 300 s.

to stimulate adenylate cyclase in these cells (e.g., parathyroid hormone or dopamine) can also inhibit the Na/H antiport (37–39). It is not clear, however, whether the mechanism of Na/H antiport inhibition in these cells is mediated through modulation of Ca_i.

Agonists that raise the Ca_i usually produce a biphasic effect on the pH_i profile in platelets and other cells. An initial and transient phase of acidification is followed by sustained alkalinization. It has been proposed that the initial phase relates to either increased metabolic activity due to the rapid rise in Ca_i, activation of Ca-ATPase (a Ca/H exchanger), or both (2, 3, 40). In this study, the treatment with PMA produced a transient acidification before alkalinization, without an apparent effect of the phorbol ester on the Ca_i. This was a puzzling finding, particularly when a PMA-evoked transient acidification was not observed in our previous study (2). The reason for the difference between the two studies is not clear, although it may relate to treatment of the platelets in this project with aspirin. It should be noted, however, that stimulation of PKC can lead to activation of Ca-ATPase and hence, transient acidification (34, 41, 42).

The relationships among Ca_i, cyclic nucleotides, and Na/H antiport may play a role in the pathophysiology of diseases such as essential hypertension and diabetes. Platelet Ca_i is elevated in essential hypertension under basal and stimulated states (43–47), a phenomenon that may reflect reduced maximal initial reaction velocity for Ca activation of the Ca-ATPase in the plasma membrane of these cells (48). Moreover, platelets from essential hypertensives exhibit lower cAMP levels (49). These observations suggest an explanation for increased platelet Na/H antiport activity in essential hypertension (50–52).

Likewise, a recent study has demonstrated that diabetes in the rat is expressed by an alkaline shift of the Xi in hepatocytes (53), a pertubation that may also relate to an imbalance between Ca_i and cyclic nucleotides.

In conclusion, the present work demonstrates that cyclic nucleotides modulate the Na/H antiport through Ca_i. It also confirms our previous observation that Ca_i is paramount to agonist and nonagonist-mediated activation of the Na/H antiport in human platelets, exerting its effect both on the Xi and Zi.

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