Thrombin Induces a Calcium Transient That Mediates an Activation of the Na⁺/H⁺ Exchanger in Human Fibroblasts*

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The calcium dependence of growth factor-induced cytoplastic alkalinization was determined in serum-deprived human fibroblasts (WS-1 cells). Intracellular pH (pHi) and intracellular calcium (Ca²⁺) were measured using the fluorometric dyes 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein and fura2, respectively. Thrombin (10 nm) induced an alkalinization (0.18 ± 0.01 pH units, n = 23) that was Na⁺-dependent and amiloride-sensitive, suggesting that the alkalinization was mediated by the Na⁺/H⁺ exchanger. Thrombin treatment caused a transient increase in Ca²⁺, (325 ± 39 nm, n = 12) that preceded the observed increase in pH. The increases in Ca²⁺ and pH were dependent on the concentration of thrombin. The thrombin-induced increase in Ca²⁺ occurred in the absence of external calcium indicating that thrombin released calcium from internal stores. Inhibition of the thrombin-induced increase in Ca²⁺, with 8-diethylaminoctoyle 3,4,5-trimethoxybenzoate hydrochloride or bis-(o-aminophenoxo)ethane-N,N',N'-tetraacetic acid also inhibited the thrombin-stimulated increase in pH.

The calcium ionophore ionomycin was used to increase Ca²⁺, independent of growth factor stimulation. When Ca²⁺ was elevated with ionomycin, a concomitant increase in pH was observed. The increase in pH due to ionomycin was dependent on Na⁺ and sensitive to amiloride. The removal of external Ca²⁺ inhibited the ionomycin-induced elevation of both Ca²⁺ and pH. The ionomycin-induced increases in Ca²⁺ and pH were not inhibited by 8-diethylaminoctoyle 3,4,5-trimethoxybenzoate hydrochloride. The results suggest that thrombin treatment can activate the Na⁺/H⁺ exchanger, and this activation is mediated by an increase in Ca²⁺.

The binding of a mitogen to its cell surface receptor activates a number of early events including the hydrolysis of phosphoinositides (PI turnover), the elevation of intracellular calcium, and an intracellular alkalinization. In order to study the mechanisms of mitogen-induced signal transduction, we have utilized human fibroblasts of fetal skin origin (WS-1 cells) as a model system. Unlike many of the immortal rodent cell lines used for similar studies, human diploid fibroblasts senesce at high passage number (1). Previous work has demonstrated that WS-1 cells can be growth-arrested by incubation for 2 days in serum-free medium (2). Adding 10 nm thrombin causes the turnover of phosphoinositides, a cytoplasmic alkalinization, and a mitogenic response as measured by increased DNA synthesis (2). This report examines the effect of thrombin stimulation on intracellular calcium (Ca²⁺) and the relationship between changes in Ca²⁺ and intracellular pH (pHi).

A variety of mitogenic agents (serum, platelet-derived growth factor, bombesin, and others) transiently elevate Ca²⁺, (3–6). Growth factor stimulation of PI turnover is thought to mediate this release of calcium from internal stores via the production of inositol 1,4,5-trisphosphate (3, 7). An alternative pathway for increasing Ca²⁺, is suggested by studies using EGF. Stimulation with EGF results in the uptake of external calcium in some cell types (8, 9).

An intracellular alkalinization mediated by the Na⁺/H⁺ exchanger occurs in a variety of cells following growth factor stimulation (10–12). In the absence of bicarbonate, the activation of the Na⁺/H⁺ exchanger by growth factors raises the pH to a level permissive for DNA synthesis (13, 14).

The transient Ca²⁺ rise induced by a number of growth factors precedes the increase in pH (15). The relationship between the two responses is not well understood, but three hypotheses have been proposed: 1) the observed rise in Ca²⁺ is necessary for the activation of the Na⁺/H⁺ exchanger (11, 16); 2) the increase in pH is independent of the rise in Ca²⁺ (5, 12, 17); or 3) a certain level of intracellular free calcium is necessary but not sufficient to induce the pH increase (18, 19). The discrepancies concerning the role of calcium in the activation of the Na⁺/H⁺ exchanger suggest the existence of several pathways for the activation of the Na⁺/H⁺ exchanger within a single cell line (8, 20). Furthermore, the expression of different pathways may vary in closely related cell types (21).

In order to investigate the relationship between mitogen-stimulated increases in Ca²⁺ and pH, thrombin was used in growth-arrested WS-1 cells to demonstrate the following: 1) thrombin increases both Ca²⁺ and pH in a concentration-dependent fashion; 2) ionomycin-induced elevation of Ca²⁺ results in cellular alkalinization; 3) both thrombin and ionomycin-induced alkalinization are due to the activation of the Na⁺/H⁺ exchanger; and 4) inhibition of thrombin or ionomycin-induced increase of Ca²⁺ inhibits the increase in pH. These data represent the first demonstration of a thrombin-induced release of calcium from internal stores in human fibroblasts.
fibroblasts and suggest that this rise in Ca\(^{2+}\) can mediate the activation of the Na\(^+/H^+\) exchanger in WS-1 human fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pentaoacetoxymethyl ester of BAPTA (BAPTA-AM), the tetracetoxymethyl ester of BCECF (BCECF-AM), and TMBS were purchased from Molecular Probes (Eugene, OR). Fura 2 pentaoacetoxymethyl ester (fura2-AM) and ionomycin were obtained from Cabiochem. Human thrombin (3000 NIH units per mg of protein) was obtained from Sigma. Amiloride was a gift from Merck, Sharp and Dohme.

**Cell Growth and Arrest**—WS-1 cells are human diploid fibroblasts of fetal skin origin (1). Stock cultures were grown on 100-mm dishes in HEPES-buffered minimal essential media supplemented with 10% fetal bovine serum. Only rapidly growing cells of passage numbers 24 through 29 were used. Experimental cultures were plated at high density (6 × 10^4 cells/cm^2) on 10-mm glass chips in 35-mm dishes with 3 ml of minimal essential media.

**Measurement of Intracellular pH**—After 2 days of growth arrest, the cells were loaded with the pH-sensitive dye BCECF by placing the chip in 3 ml of 1 μM BCECF-AM for 35 min. After washing with NHB, the chip was placed in a holder designed to fit in a standard polystyrene cuvette (22) which was inserted into a water-jacketed cuvette holder in an Aminco SLM 8000C Spectrofluorometer (SLM Instruments, Urbana, IL). A syringe connected to the chip holder with Tygon tubing was used to inject solutions without any interruption of the fluorescence measurements. The chip holder was designed to allow the perfusion of a solution from the bottom of the cuvette and the removal by a suction line at the top of the cuvette. All buffer solutions were maintained in a water bath at 37 °C. Growth factors and/or inhibitors were added to 10 ml of NHB immediately prior to injection. Ten ml of a solution could be injected within 10 s and allowed for the full exchange of the solution in cuvette. Solutions were maintained in a water bath at 37 °C. Growth factors and/or inhibitors were added to 10 ml of NHB immediately prior to injection. Ten ml of a solution could be injected within 10 s and allowed for the full exchange of the solution in cuvette.

The ratio of fluorescence (R0 = λ504/λ440) at the pH-sensitive excitation wavelength of 504 nm versus the pH-insensitive wavelength of 440 nm was measured at an emission wavelength of 530 nm. The emissions at each excitation wavelength were averaged over a 1-s interval and ratios were constructed at 2-s intervals. The fluorescence was not corrected for light scattering and autofluorescence as these values amounted to less than 5% of the value of dye-loaded cells. At the end of an experiment, R0 was calibrated using the high potassium and nigericin procedure (145 mM KCl and 5 μg/ml nigericin) described by Thomas et al. (23). After each experiment, R0 was measured at two pH values ranging from 6.8 to 7.5; all pH values were determined at 37 °C. Over this range, R0 and pH were linearly related according to the least squares line: pH = 5.84 + 0.02 R0 (R = 0.99). This equation was used to convert R0 to pH. A similar calibration curve was obtained in the presence of 1 mM amiloride, suggesting that no correction was necessary when using amiloride-containing solutions.

**Measurement of Intracellular Calcium**—The cells were grown on glass chips and growth-arrested as in the pH experiments. Following growth arrest, cells were loaded with 1 μM fura2-AM for NHB for 35 min. Loading was performed at 21 °C to minimize endocytosis of the dye and the preferential labeling of endosomal compartments (24). After loading, the cells were rinsed for 15 min in NHB at 37 °C and placed in the spectrofluorometer as in the pH experiments described above. Fluorescence was measured at a constant emission wavelength of 510 nm. Excitation wavelengths alternated between 340 nm (the peak for calcium-bound fura2) and 380 nm (the peak for free fura2). The emission for each excitation wavelength was averaged over a 1-s period. Autofluorescence at both wavelengths was measured at the start of each experiment using unlabeled cells on a glass chip. These values were subtracted from the fluorescence recordings prior to the calculation of the 340/380 nm fluorescence ratio (R0). R0 values were recorded at 2-s intervals. Following the experimental treatment, each chip was calibrated using the calcium ionophore ionomycin in the absence of calcium (NHB with no added calcium and 10 mM EGTA) to yield a minimum 340/380 ratio (R0) and a maximum 340/380 ratio (Rmax) which were used to calculate the maximal calcium and pH responses. In the case of pH, an average maximal reading maintained for at least 120 s was determined during an observation period that was limited to the first 10 min following thrombin or ionomycin stimulation. The calcium response was much more rapid than the pH response. The maximal Ca\(^{2+}\) response was obtained by averaging the maximal Ca\(^{2+}\) occurring over a 10-s period within 1 min of stimulation. The difference between the average maximal response and the average base line was calculated and used as the maximal stimulated change for that chip of cells.

The pretreatment of the chips with TMBS or with EGTA was accomplished by the perfusion of the appropriate solution through the cuvette containing the chip while in the spectrofluorometer. In all cases, a stable base line was obtained prior to stimulation with either thrombin or ionomycin. In the case of the calcium chelator BAPTA, the cells were preincubated with 30 μM BAPTA-AM for 1 h in a small Petri dish. During the final 35 min of BAPTA-AM loading, the cells were also loaded with either 2 μM fura2-AM or 2 μM BCECF-AM. The cells were rinsed in NHB for a 45-min period prior to the start of the experiment.

**RESULTS**

**Thrombin Effects on Ca\(^{2+}\) and pH.**—Exposure of growth-arrested WS-1 cells to 10 nM thrombin generated a transient increase in Ca\(^{2+}\), (Fig. 1A). The thrombin-stimulated increase in Ca\(^{2+}\) was transitory with peak values generated within 20 s from the start of thrombin addition. The peak Ca\(^{2+}\) values lasted for 6–10 s and were followed by a decrease in Ca\(^{2+}\); to below base-line levels. Ca\(^{2+}\) returned to the initial base line after 5–7 min. Thrombin addition also caused a change in pH, that was detectable within 1 min and complete within about 10 min (Fig. 2A).

The increase in Ca\(^{2+}\) and pH, was dependent on the concentration of thrombin. Table I shows the increase in Ca\(^{2+}\) and pH above a base-line value established at the start of each experiment. The average base line for Ca\(^{2+}\) was 184 ± 12 nM (n = 48) while the average base line for pH, was 7.06 ± 0.02 pH units (n = 111). At 0.1 nM thrombin, there was a small pH change and no detectable increase in Ca\(^{2+}\). As the concentration of thrombin was increased from 0.1 to 10 nM, there was a concomitant increase in both the pH and Ca\(^{2+}\) responses. The half-maximal concentration of thrombin required for the increased pH and Ca\(^{2+}\), was similar to the concentration required for DNA synthesis and inositol phosphate accumulation (2).

The peptide bombesin caused the accumulation of inositol phosphates in WS-1 cells but was ineffective in stimulating DNA synthesis (2). Even though bombesin stimulated the accumulation of inositol phosphates (2), it did not produce a detectable increase in Ca\(^{2+}\) (Fig. 1B) and no change in pH, was observed (Table I).

**Thrombin Activation of Na\(^+/H^+\) Exchange.**—The involvement of the Na\(^+/H^+\) exchanger in the thrombin-induced alkalinization was tested by measuring the sensitivity of the pH response to amiloride or Na\(^+\)-free buffer (NHB with Na\(^+\) iso-osmotically replaced by N-methyl-D-glucammonium). Exposure to Na\(^+\)-free solution caused an acidification of 0.08 ± 0.02 pH units in 100 s (n = 4) (Fig. 2B). A slower acidification was observed with 1 mM amiloride, 0.03 ± 0.01 pH units in 100 s (n = 4) (Fig. 2C). The addition of 10 nM thrombin did...
not cause an alkalinization under either of these conditions (Fig. 2, B and C). In the presence of thrombin, Na⁺-free solutions caused a rapid acidification of 0.16 ± 0.02 pH units over 100 s (n = 5). In contrast, thrombin and 1 mM amiloride resulted in an acidification similar to that observed in the absence of thrombin, 0.04 ± 0.02 pH units over 100 s (n = 4). The amiloride and Na⁺ sensitivity of the pH response indicate that the thrombin-induced change in pH occurred by activation of the Na⁺/H⁺ exchanger.

Mobilization of Internal Calcium—The elevated Ca²⁺, induced by thrombin could not be due to increased Ca²⁺ influx or to the release of Ca²⁺ from internal stores. In order to differentiate between these possibilities, the ability of thrombin to generate an increase in Ca²⁺, in the absence of external calcium was tested. The cells were pretreated with calcium-free buffer (NHB minus Ca²⁺ and with 10 μM EGTA) for 60-100 s. Pretreatment of WS-1 cells with calcium-free buffer resulted in a decrease in Ca²⁺ (Table II). Stimulation with 10 nM thrombin in the absence of external calcium resulted in a calcium transient (Fig. 3A and Table II) suggesting that thrombin releases calcium from internal stores. In order to confirm further the source of the elevated calcium, the drug TMB8 was used. TMB8 has been shown to block the release of internal calcium in human fibroblasts (27). The addition of 200 μM TMB8 reduced the basal Ca²⁺, levels and also the thrombin-stimulated calcium transient (Fig. 3B and Table II). Taken together, these results indicate that thrombin stimulation of WS-1 cells results primarily in the release of calcium from internal stores.

Relationship between Ca²⁺, and pH—The increase in pH,
prior to the administration of 10 nM thrombin. The ApHi values were calculated using the baseline established after pretreatment and baseline Ca²⁺ values indicate the effects of each pretreatment on Ca²⁺. The stimulated Ca²⁺ values represent the average maximal stimulated Ca²⁺. Mean ± S.E.; numbers in parentheses indicate number of observations.

| Pretreatment | Base-line Ca²⁺ | Stimulated Ca²⁺ | ∆pHi |
|-------------|----------------|-----------------|------|
| TMB8        | 1 µM           | 166 ± 31 (6)    | 339 ± 40 (6)  | 0.18 ± 0.05 (4) |
|            | 100 µM         | 75 ± 16 (6)     | 258 ± 23 (6)  | 0.12 ± 0.03 (5) |
|            | 200 µM         | 63 ± 10 (4)     | 142 ± 18 (4)  | 0.05 ± 0.01 (5) |
| EGTA        | 10 µM          | 54 ± 9 (6)      | 251 ± 51 (6)  | 0.14 ± 0.03 (5) |
| BAPTA-AM    | 25 µM          | 102 ± 41 (6)    | 144 ± 46 (6)  | 0.09 ± 0.01 (5) |

Pretreatment significantly reduced the thrombin-induced ∆pHi, (P < 0.01) as determined by a t test comparing thrombin-stimulated changes in pH with and without pretreatment.

Following the addition of thrombin was measured under conditions in which the rise in Ca²⁺ was blocked by TMB8. The administration of 200 µM TMB8 reduced the base-line Ca²⁺ and the thrombin-stimulated Ca²⁺ increase (Fig. 3B). The reduction in the Ca²⁺ transient correlated with a reduction in the pH response (Fig. 4B). Smaller reductions in Ca²⁺ and pH were observed with lower doses of TMB8 (Table II). In order to confirm further the importance of Ca²⁺; in mediating the thrombin-induced increase in pH, the cell-permeable calcium chelator BAPTA-AM was used to reduce the calcium transient. Preincubation of the cells with 30 µM BAPTA-AM reduced the thrombin-stimulated increase in Ca²⁺; (Table II and Fig. 3C). BAPTA did not affect the resting pH and attenuated the thrombin response (Fig. 4C). In contrast, the presence of 10 µM EGTA in the calcium-free external medium reduced the resting Ca²⁺, but still allowed a large increase in Ca²⁺; and an increase in pH, (Table II and Fig. 4A). Therefore, the thrombin-induced pH change was primarily dependent upon elevated Ca²⁺; derived from internal stores.

Ionomycin Effects on Ca²⁺; and pH—In order to examine the effect of Ca²⁺; on pH, independent of thrombin stimulation, the ionophore ionomycin was used to elevate cytoplasmic calcium. Concentrations of 1 and 2 µM ionomycin elevated Ca²⁺; (Fig. 5A). In contrast to thrombin, the ionomycin-induced changes in Ca²⁺; were dependent upon external calcium. In the absence of external calcium (10 µM EGTA), ionomycin failed to generate a large change in Ca²⁺; (Fig. 5B). However, the ionomycin-induced changes in calcium were not markedly affected by 200 µM TMB8 (Fig. 5C). These data are quantitated in Table III.

Ionomycin increased the cytoplasmic pH of growth-arrested WS-1 cells (Fig. 6A). The increase in pH caused by ionomycin correlated with the ionomycin-induced increase in Ca²⁺; (Table III). Ionomycin treatment in calcium-free buffer reduced the pH response (Fig. 6B). Pretreatment with TMB8 raised the resting pH, but had little effect on the ionomycin-induced increase in Ca²⁺; and pH, (Fig. 6C and Table III).

To determine that the ionomycin-induced alkalinization (Fig. 7A) was due to the activation of the Na⁺/H⁺ exchanger, the changes in pH, were tested for Na⁺ and amiloride sensitivity. As with thrombin-induced alkalinization, ionomycin did not stimulate an increase in pH in the absence of Na⁺ or the presence of 1 mM amiloride (Fig. 7). Ionomycin caused an acidification of 0.22 ± 0.1 pH units in 100 s (n = 4) in Na⁺-free solution (Fig. 7B), greater than that observed in the Na⁺-free buffer control (Fig. 2B). In amiloride-containing solutions, similar rates of acidification were observed with ionomycin (0.04 ± 0.01 pH units in 100 s (n = 4), Fig. 7C) or without ionomycin (Fig. 2C). Therefore, ionomycin or thrombin induced an increase in pH, that was dependent on a rise in Ca²⁺; required external sodium, and was inhibited by amiloride.

Effect of TMB8 on Na⁺/H⁺ Exchanger—In the presence of TMB8, the thrombin-induced increases in intracellular calcium (Fig. 3B) and pH, (Fig. 4B) were both inhibited. One explanation for this correlation is that TMB8 blocks calcium release from internal stores, thereby inhibiting the activation of Na⁺/H⁺ exchange by thrombin. However, given the variety of possible cellular effects of TMB8 (28), TMB8 could inhibit another signal pathway, such as protein kinase C or have a direct inhibitory effect on Na⁺/H⁺ exchange. TMB8 does not block phorbol ester activation of Na⁺/H⁺ exchange in WS-1.
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FIG. 4. Thrombin-stimulated changes in pH, are inhibited by TMB8 and BAPTA but not calcium-free buffer. BCECF fluorescence was used as a measure of pH, as described in Fig. 2. The protocol for the administration of pretreatments was identical to that employed in Fig. 3.

A.  
Ca²⁺-free Buffer  
10 nM Thrombin  

Time (seconds)

B.  
200 μM TMB8  
10 nM Thrombin  

C.  
BAPTA  
10 nM Thrombin  

FIG. 5. Effect of ionomycin on intracellular Ca²⁺. Intracellular Ca²⁺ was measured using fura2 as described in Fig. 1. The cells were pretreated with calcium-free buffer (B) or 200 μM TMB8 (C) as described in Fig. 3. Ionomycin was administered at the times indicated, either alone (A), in the maintained presence of calcium-free buffer (B), or in the maintained presence of 200 μM TMB8 (C).

TABLE III  
Calcium requirement for the ionomycin-stimulated pH change

| Treatment       | Base-line Ca²⁺ | Stimulated Ca²⁺ | ΔpH  |
|-----------------|----------------|-----------------|------|
| 1 μM ionomycin  | 188 ± 14 (7)   | 332 ± 27 (7)    | 0.14 ± 0.02 (23) |
| 2 μM ionomycin  | 190 ± 28 (9)   | 489 ± 33 (9)    | 0.35 ± 0.06 (6)  |
| 200 μM TMB8 +   | 82 ± 20 (4)    | 307 ± 79 (4)    | 0.38 ± 0.08 (4)  |
| 10 μM EGTA +    | 69 ± 41 (4)    | 119 ± 54 (4)    | 0.08 ± 0.02 (5)* |

*Pretreatment significantly reduced the ionomycin-induced ΔpH, (p < 0.01) as determined by a t test comparing ionomycin-stimulated changes in pH, with and without pretreatment.

DISCUSSION

Thrombin is a potent mitogen for a variety of fibroblasts (29–31). Treatment of cells with thrombin initiates a number of early changes that have been associated with mitogenesis, including stimulation of PI turnover (32), cellular alkalization.

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A. 7.6
1 μM Ionomycin

B. 7.4 -
2 μM Ionomycin

C. 7.2 -
200 μM TMB8

Time (seconds)

FIG. 6. Effect of ionomycin on pHi. Intracellular pH was measured using BCECF as described in Fig. 2. The cells were pretreated with calcium-free buffer (B) or 200 μM TMB8 (C) as described in Fig. 3. Ionomycin (2 μM) was administered at the times indicated, either alone (A), in the maintained presence of calcium-free buffer (B), or in the maintained presence of 200 μM TMB8 (C). Plasmic alkalinization with a similar concentration dependence.

Growth factor-induced intracellular alkalinization is mediated by an increase in the activity of the Na⁺/H⁺ exchanger in a variety of cell types (12, 13). Amiloride, an inhibitor of Na⁺/H⁺ exchange (33), prevents the thrombin-induced alkalinization of WS-1 cells (Fig. 2C). In addition, removal of external Na⁺ converts thrombin-induced alkalinization into an acidification (Fig. 2B). Such inhibition of mitogen-induced alkalinization by amiloride and Na⁺ removal is characteristic of the involvement of Na⁺/H⁺ exchange (12, 13).

The presence of Na⁺/H⁺ exchange in these cells is also suggested by the more pronounced acidification observed in Na⁺-free solutions than in the presence of amiloride. In the nominal absence of CO₂, the predominant pH regulating transport system in most cells is Na⁺/H⁺ exchange (34). At steady state, this transport system has a low level of activity and counterbalances the acid loading processes in the cell (metabolic acid production, H⁺ influx, etc.). A slow acid drift in the presence of amiloride is a measure of the acid load on a cell (34), which is quite modest in WS-1 cells (Fig. 2C). In contrast, exposure to Na⁺-free solutions results in a rapid acidification (Fig. 2B). The most likely explanation for this increased rate of acid loading is reversal of Na⁺/H⁺ exchange (with a concomitant H⁺ influx) due to the reversal of the Na⁺ gradient. Acidification induced by reversed Na⁺/H⁺ exchange has been observed in a variety of cells (12, 35). Thrombin or ionomycin in Na⁺-free solution increased the rate of acidification (Figs. 2B and 7B), suggesting activation of Na⁺/H⁺ exchange independent of the direction of the Na⁺ gradient. A similar stimulation of Na⁺/H⁺ exchange in Na⁺-free solution was observed in EGF-treated A431 cells (35).

Bombesin, platelet-derived growth factor, vasopressin, and other peptide hormones elevate Ca²⁺, by stimulating the release of Ca²⁺ from internal stores (4–6, 15, 17). Mitogens such as EGF elevate Ca²⁺, by increasing the influx of Ca²⁺ from the external medium (8, 9). Thrombin mobilizes calcium in platelets (36) and in vascular smooth muscle cells (20). In this paper, we provide the first demonstration of thrombin-stimulated calcium mobilization in human fibroblasts. The increase in Ca²⁺; is transient (Fig. 1A) and dependent on the concentration of thrombin (Table I). The removal of external calcium does not block the thrombin-induced Ca²⁺ transient (Fig. 3A, Table II), but this transient is inhibited in a concentration-dependent fashion by TMB8 (Fig. 3B, Table II), which has been shown to block the release of Ca²⁺ from internal stores (27). This release of calcium upon thrombin exposure may be mediated by the rapid thrombin-induced increase in

FIG. 7. Na⁺ dependence and amiloride sensitivity of the ionomycin-induced increase in pHi. Intracellular pH was measured using BCECF as described in Fig. 2. A, the pH response to 1 μM ionomycin; B, the effect of Na⁺-free buffer on the pH response to ionomycin; C, the effect of 1 mM amiloride on the pH response to ionomycin. See Fig. 2 for the effect of Na⁺-free buffer or amiloride alone.
A.

![Graph A](image).

B.

![Graph B](image).

Fig. 8. Effect of TMB8 on rate of recovery of pH after a NH4Cl prepulse. Intracellular pH, was measured using BCECF as described in Fig. 2. A, the pH response to a prepulse of 20 mM NH4Cl for the indicated time; B, similar experiment as in A except in the presence of 200 μM TMB8. Time of NH4Cl treatment varied to produce similar minimal pH in control and TMB8-treated cells.

Inositol triphosphate (2), which is a potent Ca2+ releasing agent (37). The elevation of inositol triphosphate and Ca2+i occurred within the first 1–2 min of exposure to thrombin and subsided within a few minutes (Figs. 1A and 3A) (2).

An unusual feature of the thrombin-induced Ca2+i transient is that Ca2+i, actually drops below base-line levels for several minutes before returning to initial values (Figs. 1A and 3A). The basis for this effect may be a mitogen activation of Ca2+ sequestering mechanisms as well as Ca2+i transport pathways. When the Ca2+i transient is blocked by TMB8, Ca2+i, drops below base line for several minutes (Fig. 3B). In addition, a similar drop in base-line Ca2+i, has been observed following treatment with low concentrations of thrombin (0.1 nM), which fail to generate an observable calcium transient (Table I). These results suggest that Ca2+i sequestering or transport mechanisms are activated independent of elevated intracellular calcium.

In contrast to the thrombin-induced changes, bombesin had no effect on either pH, (Table I) or Ca2+i, (Fig. 1B, Table I). The inability of bombesin to induce a detectable increase in Ca2+i, was unexpected given our previous observation of inositol phosphate generation in WS-1 cells following bombesin treatment. Bombesin induced a significantly greater accumulation of inositol phosphate within 30 min of stimulation when compared with thrombin. However, a comparison of thrombin and bombesin inositol phosphate production indicated that thrombin produced larger quantities of inositol bisphosphate and triphosphate at early time points (2). The rapid and greater production of inositol triphosphate may explain thrombin’s proficiency in releasing internal calcium. The failure of bombesin to induce early ionic events correlates with its lack of mitogenic stimulation of WS-1 cells (2). These results contrast with the effects of bombesin on Swiss 3T3 cells where bombesin is mitogenic (38) and results in an increase in both Ca2+i, and pH, (15, 39).

Our data suggest that elevated Ca2+i, is important for the activation of Na+/H+ exchange. Prevention of the thrombin-induced Ca2+i increase by TMB8 or BAPTA significantly reduced the cytosolic acidification (Fig. 4 and Table II). In the absence of mitogen, an increase in Ca2+i, induced by ionomycin caused a cytosolic acidification that was insensitive to TMB8 but required extracellular Ca2+i (Fig. 6 and Table II). Therefore, WS-1 cells have a pathway of mitogen-induced activation of Na+/H+ exchange that depends on elevated intracellular Ca2+i.

Regulation of Na+/H+ exchange in other cell types includes pathways activated by cellular acidification (34), volume change (40), and protein kinase C (8, 17). Other pathways for the activation of Na+/H+ exchange probably exist in WS-1 cells. With 0.1 nM thrombin, a small (less than 0.1 pH unit over 10 min) but reproducible change in pH was observed with no detectable increase in Ca2+i, in addition, treatment of cells with BAPTA or TMB8 did not completely eliminate a pH response, while significantly reducing the calcium transient. A diacylglycerol-activated protein kinase C pathway may also be present in these cells and contribute in part to the regulation of Na+/H+ exchange. Treatment of WS-1 cells with phorbol esters does not stimulate DNA synthesis, but does stimulate Na+/H+ exchange. Taken together, our results suggest that the cytosolic acidification caused by thrombin is predominantly due to a calcium-dependent pathway, but another pathway may be evident at low thrombin concentration. Other human fibroblast cell strains have either protein kinase C or calcium/calmodulin-mediated regulation of Na+/H+ exchange (41).

The calcium requirement for activation of Na+/H+ exchange varies with cell type. Elevation of Ca2+i, by exposure to calcium ionophores has been shown to activate Na+/H+ exchange in some cells, such as HSWP human fibroblasts (16), mouse thymocytes, and Swiss 3T3 cells (15). However, in vascular smooth muscle cells (20), human platelets (36), Swiss 3T3 fibroblasts (5), and HF human fibroblasts (12), the elevation of Ca2+i, with ionophores did not activate Na+/H+ exchange. In the case of stimulation by polypeptide hormones and mitogens, the role of elevated Ca2+i, in mediating cellular acidification may vary with the cell system. For example, the prevention of mitogen-induced Ca2+i, elevation inhibited the activation of the Na+/H+ exchanger in vascular smooth muscle cells (20), Chinese hamster embry fibroblasts (8), and HSWP human fibroblasts (27). In contrast, the activation of the Na+/H+ exchanger could be observed even when the calcium transient was prevented in human platelets (36) and Swiss 3T3 cells (5). In WS-1 cells, the elevation of Ca2+i, with
ionomycin is sufficient to stimulate the Na\(^+\)/H\(^+\) exchanger. In addition, the transient elevation of Ca\(^{2+}\); caused by thrombin is necessary for the thrombin-stimulated alkalinization in these cells.

Several possible mechanisms for the effect of Ca\(^{2+}\) on Na\(^+\)/H\(^+\) exchange exist. The temporal difference between the rapid calcium response and the slower change in pH suggests that calcium is initiating a signaling cascade. Ca\(^{2+}\) could activate these cells.

bin is necessary for the thrombin-stimulated alkalinization in protein kinase C. An alternative mechanism would involve the initial calcium transient (43). Alternatively, Ca\(^{2+}\) could ulin-dependent protein kinase activity can remain active after Ca\(^{2+}\) is sufficient to stimulate the Na\(^+\)/H\(^+\) exchanger. In fusion of intracellular vesicles with the plasma membrane, thereby increasing the number of exchangers at the cell surface. The mechanism of the Ca\(^{2+}\) effect on the Na\(^+\)/H\(^+\) exchange in WS-1 cells is currently under investigation.

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REFERENCES

1. Corfield, V. A., and Hay, R. J. (1978) In Vitro (Rockville) 14, 787-794
2. Hendry, B., and Manrak, M. D. (1988) J. Cell Physiol. 136, 486-499
3. Moolenaar, W. H., Tertoolen, L. G. J., and de Laat, S. W. (1984) J. Biol. Chem. 259, 8066-8069
4. Lopez-Rivas, A., Mendoza, S. A., Nanberg, E., Sinnett-Smith, J., and Rozengurt, E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5768-5772
5. Ives, H. E., and Daniel, T. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1950-1954
6. Heikkila, R., Trepel, J. B., Cuttitta, F., Neckers, L. M., and Sausville, E. A. (1987) J. Biol. Chem. 262, 16465-16480
7. Jamieson, G. A., and Villereal, M. L. (1987) Arch. Biochem. Biophys. 252, 478-486
8. Ober, S. S., and Pardee, A. B. (1987) J. Cell Physiol. 132, 311-317
9. Hill, T. D., Kindmark, H., and Boynton, A. L. (1988) J. Cell Biochem. 38, 137-144
10. Pouyssegur, J., Chambard, J. C., Franchi, A., Paris, S., and Van Obberghen-Shilling, E. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3935-3939
11. Villereal, M. L. (1981) J. Cell. Physiol. 107, 359-369
12. Moolenaar, W. H., Tsien, R. Y., van der Saag, P. T., and de Laat, S. W. (1983) Nature 304, 645-648
13. Pouyssegur, J., Sardet, C., Franchi, A., L’Allemain, G., and Paris, S. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4832-4837
14. Chambard, J. C., and Pouyssegur, J. (1986) Exp. Cell Res. 164, 282-294
15. Heuketh, T. R., Moore, J. P., Morris, J. D., Taylor, M. V., Rogers, J., Smith, G. A., and Metcalfe, J. C. (1985) Nature 313, 481-484
16. Muldoon. L. L., and Villereal, M. L. (1985) Am. J. Physiol. 249, C140-C148
17. Moolenaar, W. H., Tertoolen, L. G. J., and de Laat, S. W. (1984) Nature 312, 371-374
18. Moolenaar, W. H. (1986) Annu. Rev. Physiol. 48, 363-376
19. Miyahashi, T., and Ives, H. E. (1988) J. Biol. Chem. 263, 8790-8795
20. Huang, C.-L., Cogan, M. G., Cragoe, E. J., Jr., and Ives, H. E. (1987) J. Biol. Chem. 262, 14134-14140
21. Vicentini, L. M., and Villereal, M. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8053-8056
22. Okkuma, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3321-3327
23. Thomas, J. A., Buschbaum, R. N., Zinniak, A., and Racker, E. (1979) Biochemistry 18, 2210-2218
24. Malagoli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) J. Cell Biol. 105, 2145-2155
25. Cobbold, P. H., and Rink, T. J. (1987) Biochem. J. 248, 313-328
26. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440-3450
27. Moolenaar, W. H., Tertoolen, L. G. J., and de Laat, S. W. (1984) Proc. Natl. Acad. Sci. U. S. A. 82, 8053-8056
28. Simpson, A. W. M., Hallam, T. J., and Rink, T. J. (1984) FEBS Lett. 176, 139-143
29. Rohjanelpoto, P. (1978) J. Cell. Physiol. 95, 189-194
30. Glenn, K. C., Carney, D. H., Fenton, J. W., II, and Cunningham, D. D. (1980) J. Biol. Chem. 255, 6609-6616
31. Perdue, J. F., Lubensky, W., Kivity, E., Sonder, S. A., and Fenton, J. W. (1980) J. Biol. Chem. 256, 2767-2776
32. Carney, D. H., Scott, D. L., Gordon, E. A., and LaBelle, E. F. (1985) Cell 42, 479-488
33. Benos, D. J. (1982) Am. J. Physiol. 242, C131-C135
34. Roos, A., and Boron, W. (1981) Physiol. Rev. 61, 296-434
35. Rozenberg, P., Glaser, L., Schlesinger, P., and Cassel, D. (1983) J. Biol. Chem. 258, 12644-12653
36. Zavoico, G. B., Cragoe, E. J., Jr., and Feinstein, M. B. (1986) J. Biol. Chem. 261, 13160-13167
37. Berridge, M. J., Heisop, J. P., Irvine, R. F., and Brown, K. D. (1984) Biochem. J. 222, 195-201
38. Rosengurt, E., and Sinnett-Smith, J. W. (1982) Proc. Natl. Acad. Sci. U. S. A. 80, 2936-2940
39. Mendoza, S. M., Schneider, J. A., Lopez-Rivas, A., Sinnett-Smith, J. W., and Rozengurt, E. (1986) J. Cell Biol. 102, 2223-2233
40. Steinset, S., Cohen, S., Goetz, J. D., and Rothstein, A. (1985) J. Cell Biol. 101, 269-272
41. Muldoon, L. L., Jamieson, G. A., Kao, A. C., Palfrey, H. C., and Villereal, M. L. (1987) Am. J. Physiol. 253, C219-C229
42. Owen, N. E., and Villereal, M. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3537-3541
43. Miller, S. G., and Kennedy, M. B. (1986) Cell 44, 861-870