Lowering Extracellular pH Evokes Inositol Polyphosphate Formation and Calcium Mobilization*

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Chang-extracellular pH (pH7 from 7.4 to 6.1 increased [3H]inositol bis- and trisphosphates ~10- and 5-fold, respectively, in 15 s in human fibroblasts. [H]Inositol phosphate increased less rapidly than the polyphosphates. Bradykinin similarly increased [3H]inositol phosphates. Shifting pH7 from 7.4 to 6.0 evoked a large spike in cytosolic free Ca2+ ([Ca2+]i) which was primarily caused by the release of stored Ca2+. Changing pH7 from 7.4 to 6.0 decreased cytoplasmic pH to ~7.0. Moderate decreases in intracellular pH had no effect on [Ca2+]i or 4aCa2+ efflux. Decreasing pH7 strikingly increased 4aCa2+ efflux and decreased total cell Ca2+ similarly to bradykinin. Changing pH7 from 7.4 to ~6.4 produced half-maximal effects on [Ca2+]i, 4aCa2+ efflux, and total Ca2+. Cycling pH7 between 7.4 and 6.0 produced repetitive decreases and increases in total Ca2+. Bradykinin released the Ca2+ which was reaccumulated after an acid pulse indicating that Ca2+ had returned to the hormone-sensitive pool. Decreasing pH7 also released stored Ca2+ from coronary endothelial, neuroblastoma, and umbilical artery muscle cells, but not from rat aortic smooth muscle or human epidermoid carcinoma (A431) cells. We suggest that lowering pH7 stimulates a phosphoinositidase-coupled receptor by protonating a functional group with a pKa near 6.5.

Previously, we (Smith et al., 1989a) observed that decreasing the concentration of Na+ in the external medium ([Na+]o) mobilizes cell Ca2+ in fibroblasts, smooth muscle, and endothelial cells. Decreasing [Na+]o, apparently stimulates a phosphoinositidase-coupled receptor or receptors on the cell surface (Smith et al., 1989a). Certain divalent trace metals and Fe2+ also evoke inositol lipid hydrolysis and release stored Ca2+ from the same cell types that respond to decreasing [Na+]o (Smith et al., 1989b). The order of potency of the Ca2+

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The abbreviations used are: [Na+]o, concentration of Na+ in the external medium; [Ca2+]i, cytosolic free Ca2+; DME, Dulbecco's modified Eagle's medium; EGTA, dipotassium dibasic [ethylenebis(oxyethyleneminitrol)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP1, inositol phosphate; IP3, inositol bisphosphate; IP5, inositol trisphosphate; IP6, inositol tetrakisphosphate; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); PSS, physiological salts sodium; BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein.

mobilizing metals was the same (Cd2+ > Co2+ > Ni2+ > Fe2+ > Mn2+) in all of the cell types (Smith et al., 1989b). The divalent metals appear to stimulate an extracellular site that is reversibly blocked by Zn2+ and which may be considered a "Ca2+ receptor" (Smith et al., 1989b). In studying these two unusual Ca2+ mobilizing stimuli, we observed that decreasing pH7 induces cell Ca2+ mobilization. Here we present evidence that the protonation of a critical functional group, possibly imidazolium, in a cell surface protein induces inositol polyphosphate production and mobilizes cell Ca2+. We suggest that the three diverse Ca2+-mobilizing stimuli, divalent metals, or decreasing [Na+]o, or pH7, act on the same cell surface "receptor."

EXPERIMENTAL PROCEDURES

Cell Culture—The methods for culturing the fibroblasts from human forearm skin (Smith et al., 1989a), endothelial cells from dog coronary arteries (Smith et al., 1989), and smooth muscle cells from rat aorta (Smith and Smith, 1987) were the same as reported previously. Human neuroblastoma cells (SK-N-SH) and epidermoid carcinoma (A431) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Smith et al., 1989b). Human neuroblastoma cells (SK-N-SH) and epidermoid carcinoma (A431) cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Smith et al., 1989b).

Cell pH—Fibroblasts were grown on cover glasses (12 × 18 mm) as described (Smith et al., 1989a) and incubated with 3 μM BCECF acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) in 1 ml of DMEM for 30 min at 37 °C. Then the cover glass was rinsed and incubated with 2 ml of PSS with 10 mM glucose for 30 min before placing the cover glass on the diagonal face of a triangular cuvette. Fluorescence emission at 527 nm minus the autofluorescence of a cover glass that was not loaded with BCECF was recorded at two excitation wavelengths, 500 and 440 nm, with a Deltason dual wavelength fluorometer (Photon Technology International Inc., Princeton, NJ). Autofluorescence was not greater than 5% dye fluorescence. Calibration of cytoplasmic dye fluorescence was done with a high K+ PSS (KCl substituted for NaCl) (pH 8.0 to 6.0) containing 10 μM nigerin as described (Moolenaar et al., 1984).

Other Methods—[3H]Inositol phosphates, [Ca2+]i, 4aCa2+ efflux, total cell Ca2+, cell Na+, K+, and protein were measured as described previously (Smith et al., 1989a, 1989b). The physiological salt solution (PSS) contained (in millimolar): 120 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, and 20 HEPES-Tris (pH 7.4). PIPES-PSS contained 20 mM PIPES (Sigma) instead of HEPES (United States Biochemical Corp., Cleveland, OH). The pH of PIPES-PSS was adjusted with Tris or HCl to the desired value. All incubations were at 37 °C unless otherwise indicated.

Effect of Decreasing pHo on [Ca2+]i—Changing pHo from 7.4 to 6.0 evoked a large spike in [Ca2+]i, which was very similar to that produced by bradykinin (Fig. 1). [Ca2+]i increased rapidly in response to both stimuli to a peak (~1 μM) and then fell to nearly the basal level (Fig. 1). Smaller de-
decreases in pH, evoked smaller increases in [Ca\(^{2+}\)]. Changing pH, to 6.9 increased [Ca\(^{2+}\)], to about twice the basal level (Fig. 1). Decreasing pH, from 7.4 to 6.0 increased [Ca\(^{2+}\)], from 134 ± 9 nM (n = 9) to 978 ± 52 (n = 5) in the presence of external Ca\(^{2+}\) or to 962 ± 70 nM (n = 4) in the absence of Ca\(^{2+}\). For this experiment PIPES-PSS was removed and replaced with PIPES-PSS (pH 6.0) or PIPES-PSS with 0.1 mM EGTA and no added Ca\(^{2+}\). These data indicate that the release of stored Ca\(^{2+}\) is largely responsible for the increase in [Ca\(^{2+}\)], produced by lowering external pH.

Previously, we (Smith et al., 1989) reported that bradykinin increases [Ca\(^{2+}\)], primarily by releasing stored Ca\(^{2+}\) in human skin fibroblasts. Prior stimulation of the cells with the hormone abolished the [Ca\(^{2+}\)] response to decreasing pH, (Fig. 1B). Therefore, it appears that lowering pH, releases Ca\(^{2+}\) from the same IP\(_3\)-sensitive pool as bradykinin.

Decreases in Intracellular pH Have No Effect on [Ca\(^{2+}\)], or \(^{40}\)Ca\(^{2+}\) Efflux—Changing pH, from 7.4 to 6.0 decreases intracellular pH from ~7.3 to ~7.0 (Fig. 1). Moderate decreases in intracellular pH are readily produced by exposing cells to a weak acid at constant extracellular pH as described previously for fibroblasts (Moolenaar et al., 1984) and other cell types (Roos and Boron, 1981). The addition of 5, 10, 20, or 40 mM sodium propionate (pK \(_{4.87}\)) decreases cell pH by 0.1–0.4 units (Moolenaar et al., 1984 and Footnote 3). The addition of these concentrations of sodium propionate had no effect on [Ca\(^{2+}\)], or \(^{40}\)Ca\(^{2+}\) efflux. Because moderate decreases in intracellular pH at constant pH, failed to evoke a [Ca\(^{2+}\)] spike, the protonation of an extracellular site probably triggers Ca\(^{2+}\) mobilization.

Effect of pH, on \(^{40}\)Ca\(^{2+}\) Efflux—Lowering pH, strikingly increased \(^{40}\)Ca\(^{2+}\) efflux (Fig. 2). The maximal rate of efflux evoked by decreasing pH, is similar to that produced by bradykinin (Smith et al., 1989a). Changing pH, from 7.4 to 6.4 caused the half-maximal increase in the first-order rate coefficient of efflux (Fig. 2B). The effect of pH, was independent of the buffer used. Changing pH, from 7.4 to 6.0 produced similar increases in \(^{40}\)Ca\(^{2+}\) efflux when phosphate

3 J. B. Smith, unpublished data.
or imidazole was used to buffer PSS instead of PIPES.

**Effect of Decreasing pH on Total Cell Ca²⁺, Na⁺, and K⁺**
A hallmark of the stimulation of Ca²⁺-mobilizing receptors is a rapid net efflux of a substantial amount of total cell Ca²⁺ (Bitar et al., 1986; Smith and Smith, 1987; Kojima et al., 1985; Recocchi et al., 1982; Smith et al., 1989a). To test the effect of pH on total cell Ca²⁺, 30 μl of 1 N HCl was added to cultures that had been incubated overnight with ⁴⁰Ca²⁺. Lowering pH markedly decreased total cell Ca²⁺ (Figs. 3 and 4). The maximal decrease (~60-70%) occurred 1-2 min after decreasing pH, (Figs. 3 and 4) which is similar to that produced by bradykinin (Smith et al., 1989a). Lowering pH, to 6.5 half-maximally decreased cell Ca²⁺ (Fig. 3).

The cells slowly regained much of the lost Ca²⁺ even when pH was kept at 6.0 (Fig. 4A). Changing pH back to 7.4 markedly increased the rate of recovery of total cell Ca²⁺ (Fig. 4A). After cell Ca²⁺ had returned to the basal level, a second 2-min acid pulse decreased total Ca²⁺ similarly to the first one (Fig. 4). Next, we cycled pH, between 7.4 and 6.0 to find out if total Ca²⁺ would repeatedly rise and fall in response to the pH changes. Five successive changes in pH, repeatedly increased and decreased total cell Ca²⁺ by substantial amounts (Fig. 4B). Furthermore, bradykinin decreased cell Ca²⁺ after a partial recovery from an acid pulse (Fig. 4B), indicating that Ca²⁺ had been reaccumulated by the hormone-sensitive organelle.

Decreasing pH₅ had no effect on total cell K⁺ (p = 0.615, Student’s t test). Cell K⁺ was 1.21 ± 0.06 μmol/mg protein (n = 8) after 10 min at pH₆ compared to 1.25 ± 0.04 nmol/mg (n = 12) in control cultures incubated at pH₇. Changing pH, to 6.0 for 10 min significantly increased cell Na⁺ from 0.118 ± 0.002 (n = 12) to 0.155 ± 0.006 μmol/mg protein (n = 8, p < 0.001). The cultures were incubated for 1 h in PSS containing glucose prior to changing pH, as described for ⁴⁰Ca²⁺ efflux. The lack of an effect of pH, on cell K⁺ indicates that pH₅ selectively affects cell Ca²⁺ regulation and does not generally alter permeability of the cells to cations. Exposing the cells to pH 6 for 2-10 min had no immediate effect on cell morphology, and there was no decrease in cell viability for at least 3 days after the acid treatment as judged by phase contrast microscopy or plating efficiency after detachment with trypsin.

**Effect of Decreasing pH₅ and Bradykinin on Cellular [³H]Inositol Phosphates**—Changing pH₅ from 7.4 to 6.1 for 15 s increased [³H]IP₃ and [³H]IP₁ by ~10- and ~5-fold, respectively (Fig. 5). [³H]IP increased less rapidly than the polyphosphates (Fig. 5). [³H]IP₃ increased by ~90% 30 s after shifting pH₅ (Fig. 5). Bradykinin produced similar changes in [³H]IP₃s as lowering pH₅ (Fig. 5). Neither the hormone nor the change in pH₅ affected [³H]glycerophosphoinositol (Fig. 5).
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FIG. 5. Effects of decreasing pH₄ and bradykinin on the production of [³H]inositol phosphates. The cultures (100-mm diameter, 0.8 mg of protein) were labeled for 48 h with 20 μCi of [³H]inositol (with polymer PT 6-271, Amersham Corp., United Kingdom) as described previously (Smith et al., 1989a; Smith et al., 1989b) except the volume of the medium was decreased from 10 to 5 ml. The cultures were rinsed five times with PSS and incubated for 2 h in 10 ml of PSS containing 10 mM glucose before aspirating the medium and adding PIPES-PSS (pH 6.1) or adding 100 μl of 2 μM bradykinin. Controls at 15, 30, and 90 s did not differ significantly in [³H]inositol phosphates. Control values (counts/min/culture) were: glycerophosphoinositol 2195 ± 125; IP₂, 1291 ± 44; IP₃, 170 ± 9; IP₄, 166 ± 15; IP₄, 402 ± 37 (mean ± S.E., n = 6).

FIG. 6. Effect of decreasing pH₄ on [Ca²⁺] in coronary endothelial cells in the presence and absence of extracellular Ca²⁺. The tracings are representative of seven cover glasses in the presence of external Ca²⁺ and five in the absence of Ca²⁺.

Decreasing pH₄ Mobilizes Cell Ca²⁺ in Endothelial and Neuroblastoma Cells—Changing pH₄ from 7.4 to 6.0 transiently increased [Ca²⁺] in endothelial cells cultured from dog coronary arteries (Fig. 6). [Ca²⁺] increased rapidly from 187 ± 11 nM (n = 12) to 527 ± 48 nM (n = 7) and returned to the basal level about 2 min after lowering pH₄ (Fig. 6). Removing external Ca²⁺ from the low pH buffer and adding 0.1 mM EGTA only slightly altered the [Ca²⁺] response. [Ca²⁺] increased to 489 ± 93 nM (n = 5) in response to the pH 6 containing EGTA and no added Ca²⁺ (Fig. 6).

Lowering pH₄ strongly stimulated ⁴⁶Ca²⁺ efflux in endothelial and neuroblastoma cells (Fig. 7). Changing pH₄ to 6.4 or 6.3 half-maximally increased efflux rate coefficient in the endothelial and neuroblastoma cells, respectively. Therefore, the pH₄ dependence of Ca²⁺ mobilization was almost the same in the three cell types.

DISCUSSION
A variety of external stimuli trigger phosphatidylinositol 4,5-bisphosphate hydrolysis, including hormones, neurotransmitters, spermatozoa, photons, antigens, nucleotides, and mitogens (Berridge and Irvine, 1984). Our findings indicate a decrease in pH₄ triggers cell Ca²⁺ mobilization in fibroblasts, endothelial, smooth muscle, and neuroblastoma cells (Table I). Decreasing intracellular pH failed to mobilize cell Ca²⁺. Therefore, acidifying the extracellular medium apparently triggers Ca²⁺ mobilization by protonating a functional group, possibly imidizolium, in a cell surface protein. The imidizole
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TABLE I
Responsiveness of various cell types to three stimuli that release sequestered Ca²⁺

| Cell type           | Source                        | Stimulus of cell Ca²⁺ mobilization |
|---------------------|-------------------------------|-----------------------------------|
|                     |                               | [Na⁺]_L removal | Cadmium(II)⁶ | Lowering pH₆⁶ |
| Fibroblast          | Human skin                    | +             | +            | +             |
| Fibroblast          | Human lung (WI-38)            | +             | +            | +             |
| Smooth muscle       | Human umbilical artery        | +             | +            | +             |
| Neuroblastoma       | Human brain tumor             | +             | +            | +             |
| Epidermoid carcinoma| Human skin tumor              | +             | +            | +             |
| Endothelial         | Dog coronary artery           | -             | +            | +             |
| Smooth muscle       | Rat sorta                    | -             | +            | +             |
| Fibroblasts         | Rat embryo                   | -             | -            | -             |

¹ Smith et al., 1989a and Footnote 3.
² Smith et al., 1989b and Footnote 3.
³ This report.
⁴ Ouabain abolishes the cell Ca²⁺ mobilization produced by [Na⁺]ₐ removal in epidermoid carcinoma (A431) cells in contrast to all the other cell types. Therefore, the mechanism of [Na⁺]ₐ removal-evoked Ca²⁺ mobilization is different in A431 compared with the other cell types.

Group (pKₐ 6–7) of histidine is the most common protein functional group with a pKₐ near the pH₆ (6.4) which half-maximally induces Ca²⁺ mobilization. Acidifying the external medium produced a large and rapid increase in IP₃ (Fig. 5). Because IP₃ releases Ca²⁺ from a nonmitochondrial pool in a variety of cells (Berridge, 1987), it is likely that low pH₆-evoked IP₃ production causes cell Ca²⁺ mobilization. Hormone-receptor binding triggers IP₃ production by activating a phosphoinositidase which is regulated by a G protein (Berridge, 1987; Gilman, 1987). It is unlikely that changing pH₆ directly affects either the G protein or phosphoinositidase, because neither of these proteins have membrane-spanning domains (Gilman, 1987; Katan et al., 1988).

Cell surface receptors that mediate the endocytosis of specific macromolecules cycle continuously between the plasma membrane and intracellular organelles (Goldstein et al., 1985). After internalization the receptors encounter mild acidity (pH 5.0–6.5) in endocytic vesicles and lysosomes (Yamashiro et al., 1984). The low pH of the endocytic compartments usually causes the macromolecule to dissociate from the receptor, which is essential for receptor sorting (Brown et al., 1983). DiPaola and Maxfield (1984) observed that mild acidity induces conformational changes in the receptor for epidermal growth factor in A431 cells and in the purified asialoglycoprotein receptor reconstituted in liposomes. Turkewitz et al. (1988) showed recently that a soluble fragment of the transferrin receptor that contains 95% of its external domain undergoes a reversible conformational transition and self-association below pH 6. Lowering pH₆ may trigger inositol polyphosphate production by inducing a conformational change in the ectodomain of a cell surface protein that normally encounters low pH₆ only after endocytosis.

In spite of the vigorous investigation over the past decade of the influences of stimuli that alter cell Ca²⁺ on intracellular pH (Busa and Nuccitelli, 1984; Moody, 1984), there have been relatively few studies of the effects of pH₆ on cell Ca²⁺ regulation. Kim and Smith (1987) reported that changing pH₆ from 7.4 to 6.0 decreased [Ca²⁺]ᵢ by 25% and increased [Ca²⁺] efflux by 17% from cultured chick embryo ventricular cells. The authors suggested that the shift in pH₆ may affect Na⁺/Ca²⁺ antiport activity and Ca²⁺ binding on the cell surface. Iijima et al. (1986) examined the effects of external pH on gating and permeation in Ca²⁺ channels with the whole cell configuration of the patch clamp technique. They concluded that protonation reduces the amplitude of the negative surface potential which is sensed by the gating mechanism.

Drapeau and Nachshen (1988) examined the effects of lowering internal and/or external pH on Ca²⁺ regulation in synaptosomes. They found that changing internal pH to 5.8 or external pH to 5.5, which decreased internal pH to 6.4 in 30 s, had no effect on [Ca²⁺]ᵢ, which was measured with fura-2. Changes in intracellular pH produce relatively small changes in [Ca²⁺]ᵢ which are inconsistent with respect to the direction of the [Ca²⁺]ᵢ change among different cell types (Moody, 1984). We found that changing pH₆ to 6 decreased cell pH₆ to only about 7 and that moderate decreases in intracellular pH₆ had no effect on [Ca²⁺]ᵢ or [Ca²⁺] efflux in the fibroblasts.

The response of the fibroblasts to changing pH₆ to 6.0 is remarkably similar to stimulating the bradykinin receptor in these cells. First, both stimuli caused similar changes in intracellular [³H]inositol phosphates, suggesting that both stimuli activate phosphoinositidase rather than another phospholipase (Fig. 5). Second, both stimuli cause a large spike in [Ca²⁺]ᵢ which follows a very similar time course (Fig. 1, Smith et al., 1989a). Third, both stimuli evoke a similar increase in [Ca²⁺] efflux (Fig. 2, Smith et al., 1989a). Fourth, both stimuli provoke a rapid and reversible depletion of total cell Ca²⁺, which amounts to about 5 n mole/mg protein Ca²⁺ being expelled in 60 s (Figs. 3 and 4, Smith et al., 1989a). About 200,000 Ca²⁺ pumps/cell would be required to expel Ca²⁺ at this rate (Smith et al., 1989a). The rapidity of the recovery of total cell Ca²⁺ after changing pH₆ from 6.0 to 7.4 is noteworthy (Fig. 4) and suggests that IP₃ production abruptly stops and that IP₃ is rapidly metabolized to compounds that do not activate the intracellular Ca²⁺ release channel. Comparable studies with bradykinin have not been possible because of the lack of an effective receptor antagonist.

Previously, we (Smith et al., 1989a, 1989b) observed that removing extracellular Na⁺ and certain divalent metals trigger IP₃ production and mobilize cell Ca²⁺. Table I shows the responsiveness of various cell types to the three stimuli, decreasing pH₆, decreasing [Na⁺]ᵢ, or the addition of Cd²⁺. Four different cell types, including human neuroblastoma, dog coronary endothelial, human umbilical artery muscle, and human lung and skin fibroblasts, respond to all three stimuli. Two cell types, rat embryo fibroblasts and aortic muscle cells, did not respond to any of the stimuli. One exceptional cell type, A431 cells, responded to [Na⁺]ᵢ removal, but not to Cd²⁺ or decreasing pH₆. The mechanism of cell Ca²⁺ mobilization by [Na⁺]ᵢ removal in the case of A431 cells is clearly different from that in the other cell types. In the skin fibroblasts, endothelial, and neuroblastoma cells, Ca²⁺ mobilization is
unaffected by raising intracellular Na⁺ (Smith, 1989 and Footnote 3). In contrast, raising cell Na⁺ with ouabain completely abolishes the stimulation of [Ca²⁺] efflux in A431 cells. Therefore, decreasing intracellular rather than extracellular Na⁺ appears to provoke the release of stored Ca²⁺ in A431 cells unlike the other cell types we studied. The observation that diverse cell types respond to all three stimuli without affecting the response to bradykinin! To prove that a single receptor confers responsiveness to all three stimuli will require receptor purification and reconstitution or gene cloning and expression.

Bacteria and taste cells have chemosensors for acid. Acid is a repellent for Escherichia coli (Tso and Adler, 1974). The pH dependence of negative chemotaxis in E. coli is similar to that of Ca²⁺ mobilization in the cell types we studied, but it is unclear whether the pH sensor is an intracellular or extracellular component of the bacterium. The taste intensity of acids appears to be primarily determined by proton concentration, although the anion influences the response to acid (Biedler, 1971). Kurtharan et al. (1986) observed that decreasing pH, depolarizes mouse neuroblastoma (N-18) cells; however, it is unclear whether the depolarization of N-18 cells by acid is related to chemoreception by taste cells. Recently Akabas et al. (1988) showed that a bitter substance, denatonium, transiently increases [Ca²⁺], in a subpopulation of rat taste cells. Taste reception appears to trigger the release of stored Ca²⁺ because denatonium increased [Ca²⁺], in the absence of extracellular Ca²⁺. The cell surface receptor that triggers inositol polyphsophate formation and cell Ca²⁺ mobilization in response to decreases in pH, as shown here may be structurally related to an acid taste sensor.

REFERENCES

Akabas, M. H., Dodd, J., and Al-Awqati, Q. (1988) Science 242, 1047–1050

Footnote 3. In preparation.