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Interrelationship between growth factor-induced pH changes and intracellular Ca^{2+}

(platelet-derived growth factor/phorbol esters/bombesin/bradykinin/Na^+/H^+ exchanger)

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ABSTRACT **Many mitogens cause rapid changes in intracellular pH and Ca^{2+}. We studied the patterns of pH and Ca^{2+} changes after exposure of murine fibroblasts to platelet-derived growth factor (PDGF), bombesin, phorbol 12-myristate 13-acetate (PMA), and the vasoactive peptide bradykinin. Intracellular pH and Ca^{2+} were measured by using the fluorescent dyes 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein and fura-2. Three distinct patterns of intracellular pH change were observed. (*i*) PDGF and bombesin caused a rapid (maximum change, <2 min) cytoplasmic acidification of 0.03 pH unit followed by a slower (5–10 min) alkalinization of ≈0.11 pH unit above the resting pH of 6.88. (*ii*) PMA caused alkalinization without causing the early acidification. (*iii*) Bradykinin caused rapid acidification without the slower net alkalinization. Ionomycin also caused acidification without subsequent alkalinization. All acidification responses were amiloride resistant. Patterns of intracellular Ca^{2+} response were also determined for each agent. PDGF and bombesin caused a transient increase in cytoplasmic Ca^{2+} from a resting level of 85 ± 12 nM to 190 ± 12 nM within 2 min and return to baseline within 5 min. PMA caused no change in intracellular Ca^{2+}. Bradykinin caused the most rapid (maximum response, <20 sec) increase in intracellular Ca^{2+}. For each agonist, the Ca^{2+} transient could be blocked by buffering intracellular Ca^{2+} with quin-2. In Ca^{2+}-buffered cells, PDGF, bombesin, bradykinin, and ionomycin failed to induce cellular acidification, but alkalinization responses to PDGF, bombesin, and PMA persisted. We propose that the transient acidification seen with PDGF, bombesin, and other agents is the result of increased intracellular Ca^{2+}. However, growth factor-induced alkalinization via the Na^+/H^+ exchanger is independent of changes in Ca^{2+}.**

Cellular proliferation is stimulated by the action of mitogenic agents such as platelet-derived growth factor (PDGF), bombesin (1), bradykinin (2), and phorbol 12-myristate 13-acetate (PMA). Common among the cellular responses to these and other mitogenic agents is increased turnover of inositol phospholipids (2–5), activation of kinase C (6), and changes in intracellular pH and Ca^{2+} (7–18). Although changes in pH and Ca^{2+} have been observed in many systems, it is not yet clear which of these ionic events, if any, are necessary steps in the pathway leading to DNA synthesis. One approach to studying the events leading to cell division is to compare the ionic responses to different mitogenic agents.

The intracellular pH changes that follow mitogenic activation are complex and may reflect several different H^+ transporting mechanisms. Workers in several laboratories have reported growth factor-induced cell alkalinization caused by stimulation of an amiloride-sensitive Na^+/H^+ exchanger (7–13, 15–17). In addition to alkalinization, a number of workers have also observed a brief cellular acidification that precedes the alkalinization by several minutes (11, 12, 15). Transient acidification upon exposure to a variety of mitogenic and nonmitogenic agents has been observed in fibroblasts (11, 12, 15), Ehrlich ascites tumor cells (17), neutrophils (19, 20), and sea urchin eggs (21). In all cases in which it has been studied, this acidification seems not to be due to Na^+/H^+ exchange.

In addition to pH changes, many growth factors cause rapid increases in intracellular Ca^{2+} (14, 16, 18). In this paper, we show that this increase in cell Ca^{2+} is associated with intracellular acidification. Subsequent mitogen-induced alkalinization of the cell by activation of the Na^+/H^+ exchanger is independent of changes in intracellular Ca^{2+}.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all materials were reagent grade and were obtained from Sigma. 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM), fura-2 AM, and quin-2 AM were obtained from Molecular Probes (Junction City, OR), ionomycin was obtained from Calbiochem, bovine serum albumin was fraction V, fatty acid-poor Pentex albumin (Miles, Naperville, IL), bombesin was from Peninsula Labs (Belmont, CA); PDGF was purified from human platelets and was a gift from L. T. Williams.

Cell Culture. Swiss 3T3 fibroblasts (passage 65–72, American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells for experimentation were grown to confluence in Leighton tubes (Costar, Cambridge, MA) without medium change for the 4 days prior to experimentation.

Intracellular Measurements. The 1-cm-wide plastic strip containing the confluent monolayer of cells was removed from the Leighton tubes and cut to 3-cm lengths. These plastic strips were mounted at a 60° angle in acrylic fluorescence cuvettes (1 × 1 cm) (Sarstedt, Princeton, NJ) with a magnetic stirrer below the cell strip. Cells were washed three times and maintained at 37°C in medium consisting of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 25 mM glucose, 25 mM Hepes/NaOH (pH 7.20), and bovine serum albumin (0.5 mg/ml). For pH measurements, cells were loaded with 5 μM BCECF for 20 min and washed twice with the incubation medium. Fluorescence (excitation, 440 or 505 nm; emission, 530 nm) was measured with an SLM (Urbana, IL) 4800 spectrofluorometer in steady-state mode. Background light scattering and autofluorescence was <10% of the fluorescence signal and was subtracted during data analysis. Data were recorded and stored in an IBM PC XT computer. At the end of each experiment, the dye response was calibrated by changing

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Abbreviations: PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein.

medium to a similar incubation solution, but with 140 mM KCl, 5 mM NaCl, and 10 μg of nigericin per ml. The pH of this medium was changed to 7.2, 7.0, and then 6.8, and fluorescence at 440 and 505 nm excitation was measured at each pH. Under these conditions, intracellular pH is approximately equal to extracellular pH (22). Intracellular pH was determined by comparing the ratio of 505-nm/440-nm fluorescence during experimental periods to ratios obtained during the calibration procedure.

For Ca^{2+} measurements, cells were loaded with 2 μM fura-2 for 45 min at 37°C and then washed twice with the standard incubation medium. Fluorescence (excitation, 340 or 380 nm; emission, 510 nm) was measured and stored as described above. Background light scattering and cellular autofluorescence was <20% of the fluorescence signal and was subtracted during data analysis. To determine the intracellular [Ca^{2+}], the ratio of fluorescence at 340 nm/380 nm was compared to ratios obtained after lysis of cells into medium containing either 0 or 2 mM Ca^{2+} (23).

To clamp intracellular Ca^{2+} at a fixed concentration, cells were loaded for 40 min with 120 μM quin-2 and 5 μM fura-2 in medium containing 0 mM Ca^{2+} and 4 mM EGTA. Cells were then washed with fresh dye-free medium containing 0 mM Ca^{2+} and 4 mM EGTA. Intracellular Ca^{2+} was measured by using the same extracellular buffer and with excitation at 340 nm/380 nm and an emission wavelength of 510 nm. Under these conditions, the contribution of quin-2 to the total fluorescence signal was <25%. To correct for this contribution, we recalibrated the dye as described above. To measure pH under Ca^{2+}-clamped conditions, cells were loaded for 40 min with 120 μM quin-2 and 5 μM BCECF in 0 mM Ca^{2+} medium with 4 mM EGTA. Intracellular pH measurements were made in the same medium. Quin-2 reduced the ratio of fluorescence at excitation wavelengths of 505 nm/440 nm by ≈30%. This was corrected for by recalibration of the dye as described above.

RESULTS

The changes in intracellular pH following exposure of murine fibroblasts to PDGF, bombesin, PMA, and bradykinin are shown in Fig. 1*A*. As observed by others (11), we found that PDGF caused rapid cell acidification followed by slower alkalinization to a final pH ≈0.1 unit higher than the resting intracellular pH. Subsequent additions of PDGF within 40 min of the first exposure resulted in no further pH changes. As others have shown using amiloride (11), 60 μM dimethylamiloride blocked the alkalinization phase of the pH response to PDGF but had no effect on the acidification response. Thus, PDGF stimulation in the presence of dimethylamiloride caused a small persistent cellular acidification (data not shown). The tetradecapeptide bombesin caused pH changes that were qualitatively similar to those seen after PDGF exposure, except that the onset and initial rate of cell acidification appeared to be more brisk with bombesin. The effect of dimethylamiloride on the bombesin response paralleled that seen with PDGF. PMA caused dimethylamiloride-inhibitable alkalinization but failed to evoke the acidification phase seen with PDGF. Lastly, bradykinin caused the acidification response, but the subsequent recovery was only to the baseline pH and no higher. Thus, three different patterns of intracellular pH response were delineated. Additional experiments were done to characterize these three patterns.

Prior work indicates that PDGF, bombesin, and bradykinin all cause increased turnover of phosphatidylinositol (2–5), while PMA does not (4). Since inositol triphosphate release has been associated with transient increases in intracellular Ca^{2+}, we examined the possibility that the early acidification seen with the former agents could be explained by changes in intracellular Ca^{2+}. Intracellular Ca^{2+} was measured in cells that were exposed to PDGF, bombesin, bradykinin, or PMA (Fig. 1*B*). While PMA caused no transient increase in intracellular Ca^{2+} (and no acidification), the three agents that caused early cellular acidification (PDGF, bombesin, and bradykinin) all caused temporally coincidental transient increases in Ca^{2+}.

To examine the temporal relationship between the observed pH and intracellular [Ca^{2+}] changes in greater detail, cells were exposed to PDGF or bradykinin and studied with improved time resolution (Fig. 2). Bradykinin caused an immediate rapid increase in Ca^{2+} and a decrease in pH, while PDGF caused a slightly delayed and slower increase in Ca^{2+} and a slow decrease in pH. To see if the temporal correlation between these Ca^{2+} and pH changes might reflect a causal relationship, we examined the effects of induced cell pH changes upon cell Ca^{2+} and of induced cell Ca^{2+} changes upon cell pH.

Primary changes in cell pH were induced by transient exposure of cells to NH_4Cl followed by washing (Fig. 3). This procedure causes rapid alkalinization, followed by slow correction of cell pH toward the resting pH. After the wash,

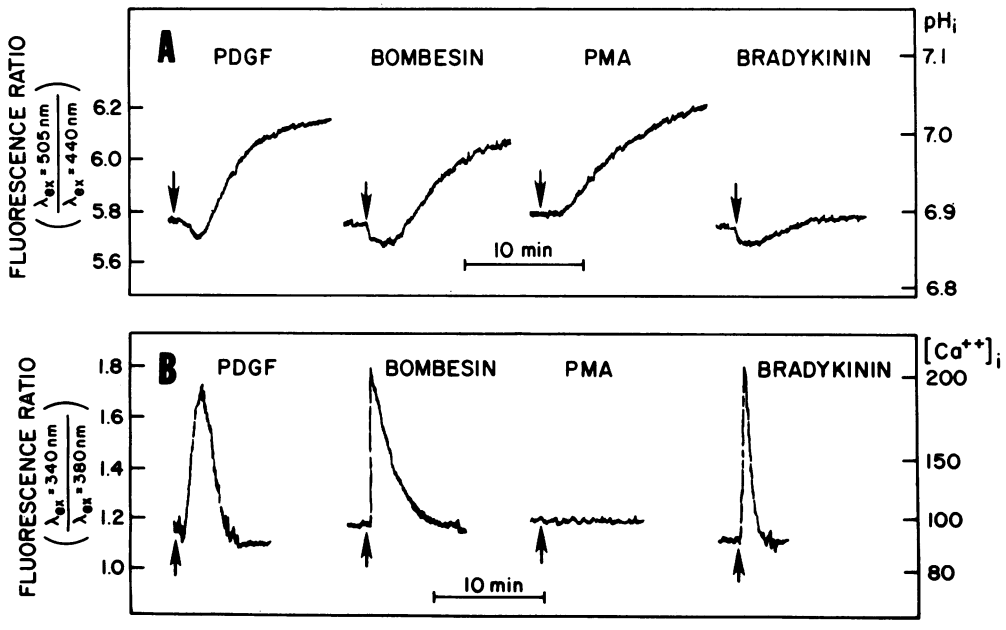

FIG. 1. Intracellular pH and Ca^{2+} (pH_i and $[Ca^{++}]_i$) changes after exposure to mitogens. (*A*) BCECF-loaded cells were exposed to 7.5 nM PDGF, 1.5 nM bombesin, 0.1 μM PMA, or 0.1 μM bradykinin at the indicated times. PDGF was electrophoretically pure; in other experiments, partially purified PDGF gave identical results. Fluorescence ratio was measured and pH was calculated as described. (*B*) In separate experiments, fura-2-loaded cells were exposed to the same concentrations of each agonist. Fluorescence ratio was measured and intracellular Ca^{2+} (indicated in nM) was calculated as described. For both *A* and *B*, results shown are typical of five to seven similar experiments.

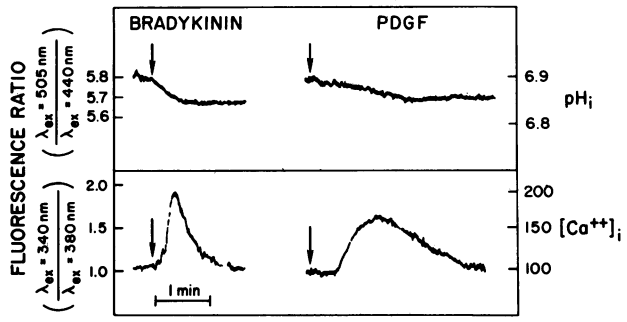

FIG. 2. Demonstration of temporal relationship between changes in intracellular pH and Ca^{2+}. Intracellular pH and Ca^{2+} (in nM) were measured as described in Fig. 1. As indicated by the bar, time resolution was increased 5-fold. Results are typical of three similar experiments.

cell pH rapidly falls and then corrects slowly. Despite these changes in intracellular pH (of similar magnitude to those following exposure to mitogens), no change in intracellular Ca^{2+} was observed.

Primary changes in cell Ca^{2+} were induced by exposure to the Ca^{2+} ionophore ionomycin (Fig. 4). Ionomycin (2 μM) caused a transient increase in intracellular Ca^{2+} similar in magnitude to that observed with the agonists described above (Fig. 4*A*); simultaneously, it caused cell acidification similar to that seen with PDGF, bombesin, and bradykinin (Fig. 4*B*). Since ionomycin is capable of transporting cations other than Ca^{2+}, several considerations were addressed. First, ionomycin has been reported to exchange Ca^{2+} for H^+ (24). However, this action would result in H^+ extrusion and cell alkalinization, which is the opposite of what was observed. Since ionomycin did not appear to catalyze Ca^{2+}/H^+ exchange in these experiments, it is possible that electroneutrality was preserved by Mg^{2+} or K^+ efflux from the cells in response to Ca^{2+} entry. Since ionomycin can transport K^+ under certain conditions (24), it was also possible that the observed cell acidification was the result of ionomycin-induced K^+/H^+ exchange. However, replacement of the extracellular medium with one containing 150 mM KCl did not alter the response to ionomycin (Fig. 4*C*), making this latter possibility unlikely. Lastly, ionomycin produced no change in intracellular pH when intracellular Ca^{2+} was clamped at a fixed level using intracellular and extracellular Ca^{2+} chelators (ref. 25; Fig. 4*D*). Thus, the cellular acidification observed with ionomycin appears to be the result of Ca^{2+} entry into the cytoplasm induced by this agent.

To show that the Ca^{2+} clamping procedure (see *Materials and Methods*) effectively blocked the Ca^{2+} transients seen with growth factors and ionomycin, we measured intracellular Ca^{2+} after exposure to bradykinin, PDGF, or ionomycin in Ca^{2+}-clamped cells. While each of these agents caused a consistent transient Ca^{2+} increase in control cells, these responses were blocked in Ca^{2+}-clamped cells (Table 1).

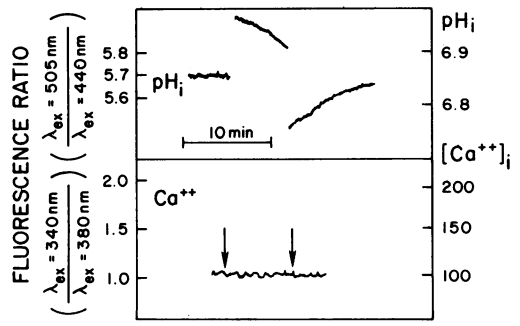

FIG. 3. Effect of intracellular pH changes on intracellular Ca^{2+}. Intracellular pH and Ca^{2+} (in nM) were measured in separate experiments as in Fig. 1. At the first arrow, 4 mM NH_4Cl was added; at the second arrow, it was removed by changing the medium. Result shown is typical of three similar experiments.

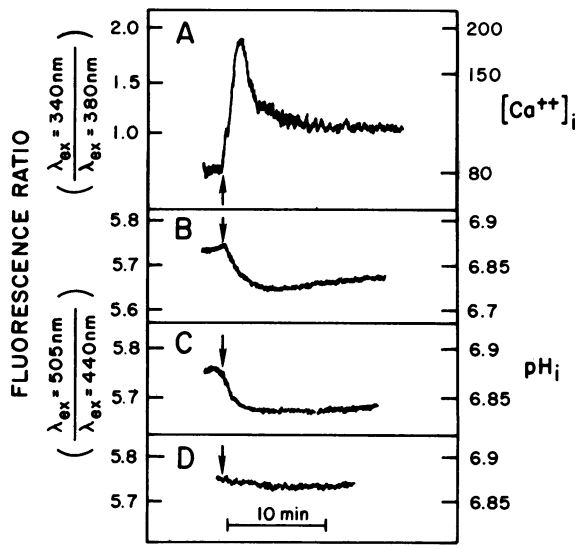

FIG. 4. Effect of ionomycin on intracellular Ca^{2+} and pH. Intracellular Ca^{2+} (in nM) was measured in fura-2-loaded cells (*A*) or intracellular pH was measured in BCECF-loaded cells (*B–D*) after exposure to 2 μM ionomycin at the indicated time. (*A* and *B*) Experiments were performed under standard conditions (see *Materials and Methods*). (*C*) Cells were incubated in medium containing 145 mM KCl and 5 mM NaCl instead of the usual concentrations of these salts. (*D*) Cells were Ca^{2+}-clamped (see *Materials and Methods*). Results shown are typical of four similar experiments.

If the agonist-induced cellular acidification truly requires increased intracellular Ca^{2+}, then eradication of the Ca^{2+} increase should prevent acidification. Indeed, in Ca^{2+}-clamped cells, intracellular pH tracings showed no early acidification response after exposure to PDGF, bombesin, and bradykinin (Fig. 5). Removal of extracellular Ca^{2+} without an intracellular chelator did not ablate the Ca^{2+} transient or the acidification seen with PDGF, bradykinin, and bombesin (data not shown), in accordance with the notion that these agents can cause release of Ca^{2+} from intracellular stores (4).

While the acidification response was ablated in Ca^{2+}-clamped cells, the alkalinization response to PDGF, bombesin, and PMA remained intact and of similar magnitude (Fig. 5) to that observed in Fig. 1*A*. In each case, this alkalinization was sensitive to 60 μM dimethylamiloride (Fig. 6).

Thus, transient intracellular acidification occurs only for agents that increase intracellular Ca^{2+}, and then only when cell Ca^{2+} increases to a measurable extent. On the other hand, activation of the Na^+/H^+ exchanger either does not require increased Ca^{2+} or it requires only a small undetectable increase in Ca^{2+}.

Table 1. Intracellular Ca^{2+} responses in control and Ca^{2+}-clamped cells

	Control	Ca^{2+}-clamped
No addition	85 ± 12	74 ± 9
Bradykinin (0.1 μM)	208 ± 17*	80 ± 7
PDGF (7.5 nM)	190 ± 12*	79 ± 10
Ionomycin (2 μM)	245 ± 38*	90 ± 13

Maximum intracellular Ca^{2+} (in nM) after addition of the indicated agents was measured in control and Ca^{2+}-clamped cells as described. *Significantly different from control; $P < 0.001$ by Student's t test ($n = 3$).

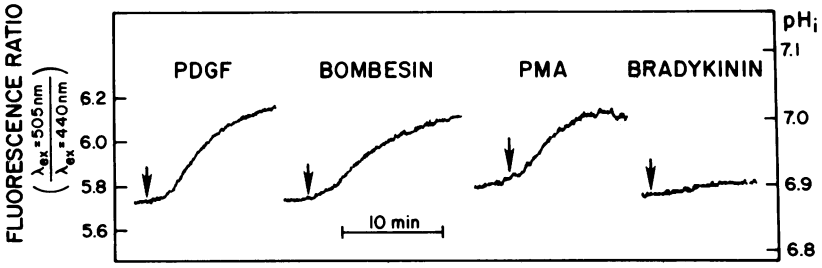

FIG. 5. Agonist-induced intracellular pH changes in Ca^{2+}-clamped cells. Cells were Ca^{2+}-clamped and intracellular pH was followed after addition of the indicated agonists. Each panel is a single experiment typical of three or four similar experiments for each agonist.

DISCUSSION

Changes in intracellular ion composition are prominent following cellular activation by mitogens. Although the precise role for these ionic changes is being actively debated (26, 27), they afford a useful tool for cataloguing and distinguishing responses to different types of mitogen. In particular, these studies were motivated by the observation that PDGF causes brief cellular acidification, while PMA does not.

The agents that we tested showed three different patterns of ionic response. The first pattern, elicited by PDGF and bombesin, is an early transient increase in cytoplasmic Ca^{2+} and a decline in cell pH followed by a later cell alkalinization. Our data suggest that the acidification is due to increased cytoplasmic Ca^{2+}, while, as previously shown, alkalinization reflects activation of the Na^+/H^+ exchanger. The second pattern, elicited by PMA, is Na^+/H^+ exchanger-induced alkalinization with no Ca^{2+} transient or acidification. The third pattern, elicited by bradykinin, is a Ca^{2+} transient and acidification without net alkalinization.

For all of the agents tested, there was one consistent relationship between intracellular Ca^{2+} and pH. The transient acidification immediately following agonist exposure appears to be the result of increased intracellular Ca^{2+}. The acidification was seen with all agents that increase Ca^{2+}, including ionomycin, was not seen with an agent that does not increase Ca^{2+} (PMA), and could be eliminated by clamping intracellular Ca^{2+} at resting levels. The ionic mechanism for this acidification response to increased Ca^{2+} has not yet been established, but several possible mechanisms can be considered.

Similar to what we observed in fibroblasts, direct injection of Ca^{2+} into snail neurons causes acidification of the cytoplasm (28). It was originally proposed that these H^+ ions are released from Ca^{2+}-binding sites upon binding of Ca^{2+} (28). Since exogenously imposed small pH changes did not alter cell Ca^{2+} in our murine fibroblasts (Fig. 5), we cannot demonstrate the expected reciprocal effect of pH on intracellular Ca^{2+}. However, a contribution of this mechanism to the acidification cannot be entirely ruled out on the basis of our data.

Since ruthenium red (a mitochondrial Ca^{2+}-channel blocker) prevents part of the acidification following Ca^{2+} injection into snail neurons, it was proposed that H^+ may in part be ejected from mitochondria following uptake of Ca^{2+} (29). Isolated mitochondria eject 1 H^+ for every Ca^{2+} that enters the mitochondrial matrix (30). Thus, it appears likely that at least part of the increase in cytosolic H^+ that we observed after increases in cytosolic Ca^{2+} arises from mitochondria.

H^+ may also be transported into the cytosol as Ca^{2+} is removed from the cytosol across nonmitochondrial membranes. ATP-dependent Ca^{2+} uptake in muscle sarcoplasmic reticulum (31), erythrocyte membranes (32, 33), and Ehrlich ascites tumor cell plasma membranes (34) is thought to be associated with oppositely directed H^+ transport. Therefore, it is possible that the transient acidification following growth factor activation results from the action of a Ca^{2+}/H^+ antiport system involved in the reuptake of Ca^{2+} into intracellular stores or the export of Ca^{2+} from the cell.

In distinction to our findings, PMA causes a transient cellular acidification in polymorphonuclear leukocytes (20). In these cells, this acidification is thought to represent activation of NADPH oxidation or the accompanying activation of the hexose monophosphate shunt. While we observed cytosolic acidification with agents that increase cytosolic Ca^{2+}, we did not observe acidification with PMA in fibroblasts, perhaps because of a difference in the metabolic pathways found in the two cell types.

Following the Ca^{2+}-induced cellular acidification, all of the agents we tested, with the exception of bradykinin (Fig. 7*A*), caused net intracellular alkalinization. Amiloride-sensitive recovery to baseline pH was observed with bradykinin (Fig. 7*B*), which most likely reflects acid-induced allosteric activation of the Na^+/H^+ exchanger (35). However, when acidification was prevented by Ca^{2+} clamping, bradykinin caused no detectable pH change (Fig. 7*C*). Taken together, these data suggest that bradykinin does not primarily activate the Na^+/H^+ exchanger.

Like many growth factors, bradykinin causes inositol phospholipid turnover (2–4), increased intracellular Ca^{2+}, and protein kinase C activation as reflected by increased expression of c-myc RNA and phosphorylation of the 80-kDa protein kinase C endogenous substrate (36). Our observation that bradykinin does not primarily activate measurable Na^+/H^+ exchange indicates that bradykinin action is distinct from that of other activators of protein kinase C. This distinction may reflect the fact that bradykinin induces protein kinase C activation to a level above the threshold for detection of some protein kinase C-mediated events but below that for measurable Na^+/H^+ exchanger activation. It is also possible that bradykinin, unlike the other agents we tested, fails to initiate an undefined process essential for activation of Na^+/H^+ exchange. In any event, the pH response to bradykinin remains an interesting anomaly, which requires further investigation.

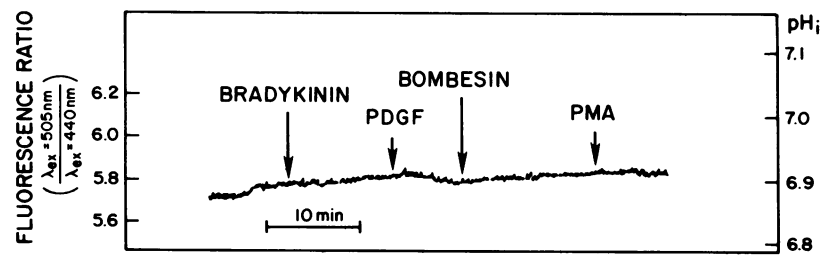

FIG. 6. Effect of dimethylamiloride on agonist-induced intracellular pH changes in Ca^{2+}-clamped cells. Cells were Ca^{2+}-clamped and exposed to 60 μM dimethylamiloride. In a single experiment typical of three similar experiments intracellular pH was measured during addition of the indicated agonists.

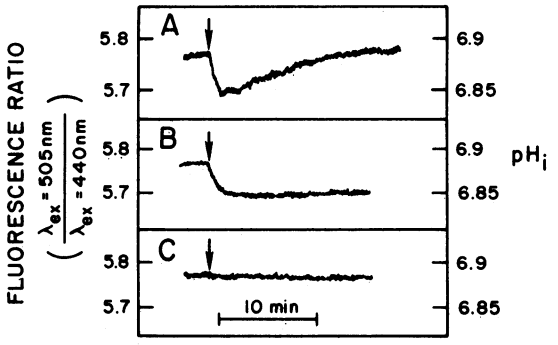

FIG. 7. Intracellular pH after exposure to bradykinin. (*A*) As in Fig. 1, BCECF-loaded cells were exposed to 0.1 μM bradykinin at the indicated time. (*B*) Cells were exposed to 60 μM dimethylamiloride 10 min before addition of bradykinin. (*C*) Cells were Ca^{2+}-clamped and then exposed to 0.1 μM bradykinin at the indicated time. Results are typical of five similar experiments.

Lastly, our data indicate that increased intracellular Ca^{2+} is neither necessary nor sufficient for activation of the Na^+/H^+ exchanger in murine fibroblasts. This is based on our finding that the Na^+/H^+ exchanger can be stimulated in the absence of measurable Ca^{2+} changes (with PMA and in Ca^{2+}-clamped cells) and that increased cytoplasmic Ca^{2+} (resulting either from bradykinin or ionomycin) does not activate the Na^+/H^+ exchanger. These findings conflict with other results, which suggest that increased intracellular Ca^{2+} is sufficient to activate the exchanger (37). Recent data indicate that Ca^{2+}-induced activation of the Na^+/H^+ exchanger in lymphocytes is mediated by changes in cell volume (38). Variability in the volume response to increased intracellular Ca^{2+} from one cell type to another may explain the observed differences in Ca^{2+} effects on activation of Na^+/H^+ exchange. As delineation of the mechanism(s) for stimulation of Na^+/H^+ exchange is an area of intense interest, further work will be necessary to determine the precise role for Ca^{2+} and protein kinase C in activation of this transport system.

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