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Alteration of Cytoplasmic Ca^{2+} in Resting and Stimulated Human Neutrophils by Short-Chain Carboxylic Acids at Neutral pH

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The results reported here indicate that the short-chain carboxylic acids acetate and propionate stimulate cytoplasmic calcium mobilization in human polymorphonuclear leukocytes, while butyrate and lactate do not. Together with the results of previous work, this indicates that there are at least three classes of short-chain carboxylic acids: those which can alter only cytoplasmic pH (e.g., lactic acid), those which can alter cytoplasmic pH and actin (e.g., butyric acid), and those which can alter cytoplasmic pH, actin, and calcium (e.g., acetate and propionate).

Short-chain carboxylic acids (SCCAs) are produced in millimolar quantities as metabolic by-products of pathogenic anaerobic bacteria (11). Work from this and other laboratories clearly demonstrates that these SCCAs effect membrane perturbations, alter cytoplasmic actin, pH, and oxygen metabolism, and can inhibit granulocyte motility (2, 3, 7–9, 15, 17, 18, 21–23, 25–27). Because alterations in cytoplasmic calcium influence all of the aforementioned cellular effects, we were interested in determining whether SCCAs can also affect cytosolic calcium. The effects of four selected SCCAs (acetate, propionate, butyrate, and lactate) were examined.

Whole blood was obtained from healthy donors by venipuncture by using Vacutainer tubes containing 1,500 U of heparin, and polymorphonuclear leukocytes (PMNs) were isolated by Mono-Poly resolving medium and Ficoll-Hypaque gradient centrifugation at 700 × *g* for 45 min at 25°C as previously described (3, 12). The neutrophil layer was removed and resuspended in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (CMF-HBSS + HEPES). The cell suspension was centrifuged at 200 × *g* for 12 min at 4°C, the erythrocytes were removed by hypotonic lysis in distilled water, and the neutrophil suspension was sedimented at 200 × *g* for 12 min at 4°C. The neutrophil pellet was resuspended at a concentration of 10^7 cells/ml in CMF-HBSS + HEPES (pH 7.4). Cell viability was determined to be >95% by trypan blue exclusion.

Intracellular free calcium concentration was determined by monitoring the fluorescent indicator Indo-1 AM (10). Neutrophils (10^7/ml) in CMF-HBSS + HEPES (pH 7.4) were incubated with 5 μM Indo-1 AM for 15 min at 37°C. The cells were washed with CMF-HBSS + HEPES and resuspended at 10^6/ml in HBSS + HEPES. Neutrophils were placed in a 1-ml cuvette, which was then placed in a spectrofluorometer (SPF-500 SLM; AMINCO) with stirring and maintained at 37°C. Fluorescence was excited at 355 nm and monitored at emissions of both 405 and 485 nm. The minimum calcium ratio following digitonin lysis was determined in the presence of 10 mM EGTA, while the maximum calcium ratio was determined in the presence of 1 mM $CaCl_2$. Calcium concentration was calculated by using the ratio of fluorescence at 405 and 485 nm (F_{405}/F_{485}) and a K_d of 250 nM. Experiments were initiated by the addition of formylmethionyl-leucyl-phenylalanine (fMLP) to HBSS, SCCA-HBSS, or EGTA-HBSS, pH 7.4. All data are reported as the means ± SEMs of at least three separate experiments. To differentiate between external calcium entry and internal calcium release, internal calcium release was determined following external calcium chelation. This was achieved by including 10 mM EGTA in the cell suspension 30 s prior to SCCA addition. Internal calcium release was calculated by subtracting the initial baseline calcium concentration from the maximal calcium response. External calcium entry was then determined by subtracting initial baseline calcium concentration and internal calcium release from total cytoplasmic calcium response. To examine the relationship of G proteins and SCCA-mediated calcium stimulation, neutrophils (5×10^6/ml) were incubated at 37°C with 500 ng of pertussis toxin (Sigma) per ml for 2 h. Indo-1 AM (5 μM) was added 15 min before the end of incubation. Neutrophils were then washed and resuspended in HBSS plus 10 mM HEPES. Control cells were incubated for 2 h at 37°C without any addition. Preliminary experiments examining the effects of SCCA on protein kinase C utilized the fluorescent probe NBD-phorbol ester (1a, 5a).

Figure 1A displays the dose effect of selected SCCAs on cytoplasmic calcium. The initial intracellular resting calcium level was 168 ± 4 nM. The data indicated that acetate and propionate elicited dose-dependent calcium increases, while butyrate and lactate elicited no calcium response. The maximum acetate- and propionate-induced responses were 473 ± 56 and 460 ± 55 nM above the baseline level, respectively. These were 59 and 57%, respectively, of the maximum response to 10 nM fMLP (804 ± 69 nM) (data not shown). The half-maximal response occurred at 2.5 mM for both acetate and propionate. A mixture of the four SCCAs induced a dose response midway between that of the two sets of SCCAs. Typical time courses of responses to fMLP, acetate, and acetate plus butyrate are displayed in Fig. 1B. Propionate and propionate plus butyrate resulted in similar time courses (data not shown). The time courses indicated that fMLP, acetate, acetate plus butyrate, propionate, and

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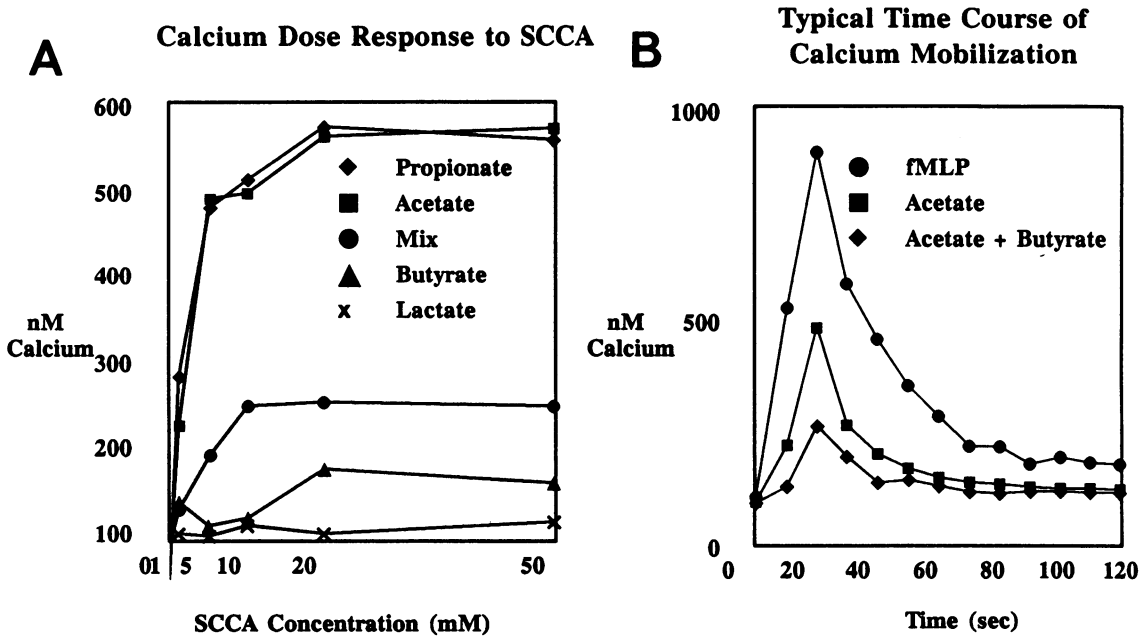

FIG. 1. (A) SCCA-induced calcium response in neutrophils. Neutrophils were stimulated with increasing concentrations of acetate, butyrate, lactate, propionate, or a mixture of these four SCCAs. The data indicated that acetate and propionate stimulated a significant calcium response in a dose-dependent manner, while butyrate and lactate did not. Each point represents the mean of three or four experiments. (B) Typical time course of PMN calcium mobilization induced by SCCAs and fMLP. Neutrophils were incubated with 5 mM acetate, an equal mixture of 5 mM acetate and butyrate, or 10 nM fMLP. The data indicated that the timing is similar in the three responses. fMLP caused the largest calcium mobilization, acetate caused an intermediate mobilization, and butyrate inhibited the acetate-induced calcium mobilization.

propionate plus butyrate all resulted in similar kinetic responses, differing only in the magnitudes of their stimulatory effects.

To identify the SCCA-stimulated calcium source, we applied the following reasoning. Addition of EGTA to the suspending buffer chelates extracellular calcium, thereby eliminating calcium entry. Therefore, in the presence of EGTA, only internal calcium release was observed. The results displayed in Fig. 2 indicated that the presence of EGTA significantly decreased, but did not eliminate the

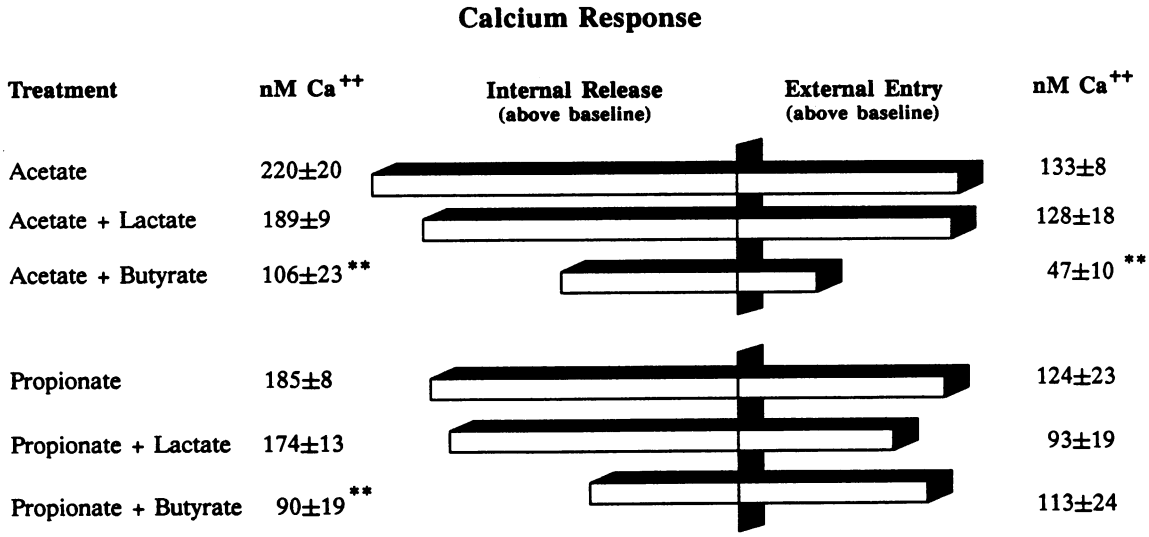

FIG. 2. Source of calcium response elicited by SCCA. The graph displays the calcium response above baseline (168 ± 4 nM). The data indicated that acetate and propionate stimulate approximately two-thirds of the calcium response from internal calcium release and one-third of the response from entry of external calcium. The maximum cytoplasmic calcium response was determined following the addition of 5 mM SCCA alone. To determine internal calcium release, EGTA was added to the suspending buffer 30 s prior to addition of SCCAs. Internal calcium release was determined by subtracting internal calcium release from total calcium response. Furthermore, while lactate had little effect, butyrate significantly inhibited acetate- and propionate-induced intra- and extracellular calcium mobilization. Values are means of three experiments.

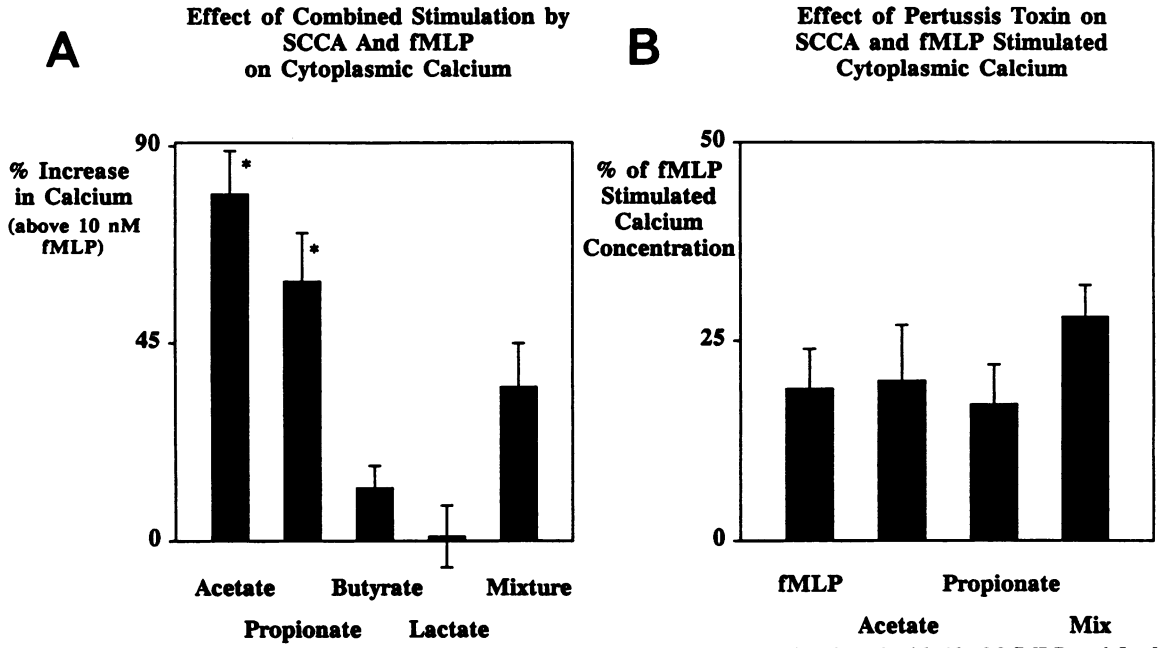

FIG. 3. (A) Effect of SCCAs on fMLP-induced calcium mobilization. Neutrophils were incubated with 10 nM fMLP and 5 mM of each SCCA. The data indicated that all four SCCAs increased the fMLP-induced calcium mobilization. However, only acetate and propionate significantly increased the calcium response, while butyrate and lactate did not. Finally, a mixture of all four SCCAs increased (but not significantly) the fMLP-induced calcium response by an amount midway between those induced by acetate and propionate and those induced by butyrate and lactate. Values are means of three experiments. (B) Effect of pertussis toxin on SCCA- and fMLP-stimulated cytoplasmic calcium. Neutrophils were incubated with 500 ng of pertussis toxin per ml for 2 h at 37°C in HBSS prior to cell stimulation. Controls were incubated with HBSS alone. The calcium response is displayed in comparison to that of the control fMLP-stimulated cells. These results indicated that both the fMLP- and SCCA-mediated calcium responses were significantly inhibited by pertussis toxin.

acetate- and propionate-stimulated cytoplasmic calcium response (decreases were 38.1% ± 3.2% and 39.1% ± 5.8% for acetate and propionate, respectively [both $P < 0.01$ compared with the effect of acetate or propionate alone). This indicated that both acetate and propionate elicited significant calcium increases of external and internal origin and that slightly more than one-third of the calcium came from extracellular entry. To determine whether lactate and butyrate inhibited entry of extracellular calcium, intracellular calcium release, or both, we carried out the following experiments. Butyrate or lactate was added to acetate or propionate in the presence or absence of EGTA. In the presence of butyrate and EGTA, the acetate- and propionate-stimulated calcium responses were significantly decreased by 70.8% ± 5.4% and 70.9% ± 6.0%, respectively ($P < 0.01$ compared with the effect of acetate or propionate alone). These values were also significant ($P < 0.05$) compared with those obtained by the addition of EGTA to acetate or propionate. In contrast, lactate and EGTA plus either acetate or propionate significantly reduced the calcium response (46.7% ± 2.7% and 43.3% ± 4.4%, respectively; however, $P > 0.05$ compared with the effect of EGTA alone). These results indicated that acetate and propionate stimulated increases in cytoplasmic calcium from both intra- and extracellular sources. They also indicated that butyrate (but not lactate) inhibited both intracellular release and extracellular entry of calcium stimulated by acetate. In contrast, butyrate inhibited only internal calcium release stimulated by propionate.

We next examined the effect of SCCA on fMLP-induced calcium response. Figure 3A displays the results of these experiments. Acetate and propionate significantly increased the fMLP-mediated calcium response by 79.0% ± 10.0% and 59.0% ± 11.4%, respectively ($P < 0.01$ and $P < 0.05$, respectively, compared with the effects of fMLP alone (percent increase = $\{[(R_{fMLP} + R_{SCCA}) - R_{fMLP}]/R_{fMLP}\} \times 100$, where R is the elicited calcium response). In contrast, butyrate and lactate increased the fMLP-stimulated calcium response by 12.5% ± 5.0% and 0.8% ± 7.0%, respectively, which was not statistically significant (both $P > 0.05$). The effect of the SCCA mixture was midway between that of calcium-releasing SCCAs and that of the non-calcium-releasing SCCAs (35.5% ± 10.5%) ($P > 0.05$). It is interesting that the combined calcium response elicited by the mixture of acetate and fMLP or the mixture of propionate and fMLP is greater than the effect calculated from the mathematical addition of the calcium responses elicited by acetate plus fMLP (39.9% ± 10%) and by propionate plus fMLP (31.7% ± 9.6% increase above fMLP alone). These results indicated that both acetate and propionate synergistically enhanced the fMLP-mediated calcium response.

These results present an apparent paradox. The work described here indicated that propionate significantly enhanced fMLP-induced calcium mobilization. In contrast, our previous work (3) indicated that propionate significantly inhibited fMLP-stimulated cytoskeletal actin oscillations and cell polarization. Furthermore, this propionate-mediated actin effect was inhibited by pertussis toxin. We therefore examined the effect of pertussis toxin on SCCA-mediated calcium mobilization. The results displayed in Fig. 3B indicate that pertussis toxin significantly inhibited both fMLP- and SCCA-stimulated calcium mobilization to approximately 25% of that stimulated by fMLP alone. This suggests that acetate and propionate operate through G proteins.

Because of the similarity of the SCCA-mediated responses to those elicited by fMLP, we examined the effect of SCCA on protein kinase C. Preliminary results indicated that lactate and butyrate had little effect on baseline protein kinase C activity. However, acetate and propionate stimulated protein kinase C activity to levels approximating that achieved by fMLP (data not shown).

The results of these studies provide three novel findings. First, some (e.g., acetate and propionate) but not all (e.g., butyrate and lactate) SCCAs can stimulate cytoplasmic calcium increases from both internal and external sources. Second, butyrate can partially inhibit this calcium response. Third, acetate and propionate, but not butyrate or lactate, can act in synergy with fMLP to increase cytoplasmic calcium. Because alterations in cytoplasmic calcium are crucial for cell function (13, 14, 16, 20), these results, together with our previous work (3, 15), have two broad implications regarding SCCAs. First, they call into question the use of some SCCAs as pure hydrogen ionophores for the specific and isolated purpose of altering cytoplasmic pH (4). Conversely, the results also indicate that lactate may be a good SCCA for this specific purpose (3, 15). Second, they begin to suggest mechanisms whereby some SCCAs can alter polymorphonuclear leukocyte functional capacity. For example, by activating calcium release, acetate and propionate may prime polymorphonuclear leukocytes for some functions, such as the generation of H_2O_2 (18, 26). However, in stimulating alterations in cytoplasmic pH, actin, and calcium, acetate and propionate may also inhibit or alter other polymorphonuclear leukocyte functions, such as chemotaxis and phagocytosis (2, 3, 8, 9).

Regarding the site of SCCA-mediated action, we noted that EGTA decreased but did not eliminate acetate- and propionate-stimulated calcium mobilization. Therefore, this cytoplasmic calcium increase derived from both intra- and extracellular sources. Interestingly, while butyrate inhibited acetate- and propionate-induced calcium mobilization, it did not inhibit fMLP-stimulated calcium responses. Conversely, acetate and propionate significantly enhanced fMLP-induced calcium mobilization. These findings support the concept that the site of SCCA effect is downstream from the fMLP receptor (3, 17). This is supported by the observation that pertussis toxin inhibits SCCA-mediated calcium mobilization. The results also suggest that SCCAs utilize similar mechanisms to stimulate increases in cytoplasmic calcium and that these mechanisms may be both similar to and distinct from those utilized by fMLP.

Finally, the current data, together with previous work (3, 15), suggest that there are at least three classes of SCCAs. The first class is typified by lactate, which can stimulate cytoplasmic pH oscillation but is not sufficient to mobilize cytoplasmic actin or calcium. The second class is typified by butyrate, which can stimulate both cytoplasmic actin and pH oscillation but is not sufficient to mobilize cytoplasmic calcium. The third class is typified by acetate and propionate, which can stimulate oscillations of pH, actin, and calcium. These results also support the concept that alterations in cytoplasmic pH, actin, and calcium can occur independently of one another (1, 3, 6, 18, 24).

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