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PERTUSSIS TOXIN INHIBITS INTRACELLULAR pH CHANGES IN HUMAN NEUTROPHILS STIMULATED BY N-FORMYL-METHIONYL-LEUCYL-PHENYLALANINE

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SUMMARY: Changes of intracellular pH in human neutrophils were monitored by 9-aminoacridine fluorescence. Both initial acidification and subsequent alkalinization phases induced by a chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine were dependent on the extracellular Ca^{2+}-concentrations, and a calcium ionophore, A-23187 similarly induced the pH-changes. Pertussis toxin inhibited the pH-changes induced by the peptide while cholera toxin did not. The pH-changes induced by A-23187 were not affected by the toxins. The results suggest that the inhibitory guanine-nucleotide regulatory protein and Ca^{2+} are involved in the pH-changes induced by the peptide.

Induction of intracellular pH (pHi) changes in various stimulus-response coupling systems has been documented (1). Sha'afi et al. (2, 3) and Grinstein and Furuya (4) have shown that a biphasic pHi change occurs in neutrophils on stimulation by a chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP) which causes chemotaxis, aggregation, degranulation and respiratory burst in the cells. They stated that the alkalinization phase of the pHi changes is likely to be mediated by Na^+/H^+ exchanger, based on the inhibitory effect of amiloride known to block the exchange reaction (5).

Two guanine nucleotide-binding regulatory components of the adenylate cyclase system (Ns and Ni) have been studied in detail (6, 7): the former is ADP-ribosylated by cholera toxin and the latter by pertussis toxin. Two groups of investigators (8, 9) showed that the treatment of guinea pig neutrophils with pertussis toxin results in inhibitions of FMLP-mediated

Abbreviations used: FMLP, N-formyl-methionyl-leucyl-phenylalanine; Ns and Ni, the guanine nucleotide regulatory proteins responsible for stimulation and inhibition of adenylate cyclase, respectively; pHi, intracellular pH; 9-AA, 9-aminoacridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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reactions: release of arachidonic acid, degranulation and generation of superoxide. Furthermore, the toxin is inhibitory to other FMLP-induced functions of neutrophils such as a rise in intracellular free Ca^{2+} (10, 11), a breakdown of phosphatidylinositol 4,5-bisphosphate (11, 12), chemotaxis and aggregation (13). The inhibitory effects seem to be closely related to the ability of the toxin to catalyze the ADP-ribosylation of Ni (8, 9).

In this paper, we show that pertussis toxin inhibits the pHi changes induced by FMLP in human neutrophils, while it does not inhibit the changes induced by A-23187. Possibilities are discussed that the intracellular free Ca^{2+} is involved in the pHi changes and that Ni may regulate the pHi changes induced by FMLP.

EXPERIMENTAL

Human neutrophils were prepared from healthy adult donors by dextran sedimentation, hypotonic hemolysis and the Conray-Ficoll method as previously described (14). Neutrophils were suspended at $2x10^7$ cells/ml in an incubation-medium (135 mM NaCl, 5 mM KCl, 2 mM glucose, 20 mM Hepes, pH 7.4) and pretreated with pertussis toxin(0.1-2.0 μg/ml) or cholera toxin(2.0 μg/ml) at 37°C for 2 hours. Control and toxin treated cells were washed twice and then suspended in NaCl-medium (140 mM NaCl, 1 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose, 10 mM Hepes, pH 7.3) for pHi measurements. In choline-medium, NaCl was replaced by choline chloride. Cell viability after the toxin treatment was greater than 90% as assessed by exclusion of trypan blue.

pHi measurements were performed by measuring fluorescence changes of 9-aminoacridine (9-AA) using a Hitachi fluorescence spectrophotometer 650-40 equipped with a thermostatically controlled cuvette holder. The excitation and emission wavelengths were 400 and 456 nm, respectively, with 5-nm bandwidths. Cationic form of 9-AA does not permealize the cells but neutralized 9-AA permealizes the cell and the quenching of the fluorescence occurs immediately. Therefore, the decreased fluorescence represents an acidification and the increased fluorescence represents an alkalinization. In a typical experiment, toxin-treated or control cells were suspended in NaCl-medium($3x10^7$ cells/ml) and incubated for 10 minutes at 37°C with 4 μM 9-AA, then stimulants were added and the fluorescence changes were continuously recorded. FMLP and A-23187 had no effects on the fluorescence of 9-AA.

Amiloride was the gift of Merck Sharp and Dohme Research Lab., West Point, USA. Pertussis toxin was the gift of Chemo-Sero Therapeutic Research Institute, Kumamoto, Japan and cholera toxin was purchased from the Institute. 9-AA was from Nakarai Chem. Co., Kyoto, Japan, FMLP from Sigma Chem. Co., St. Louis. Mo, USA and A-23187 from Calbiochem-Behring Co., San Diego, USA. Other reagents were of analytical grade.

RESULTS AND DISCUSSION

Addition of FMLP to neutrophils in NaCl-medium induced a biphasic pHi change; an initial acidification and a subsequent alkalinization which exceeded the pHi level of the resting state (Fig. 1A). After the acidifica-

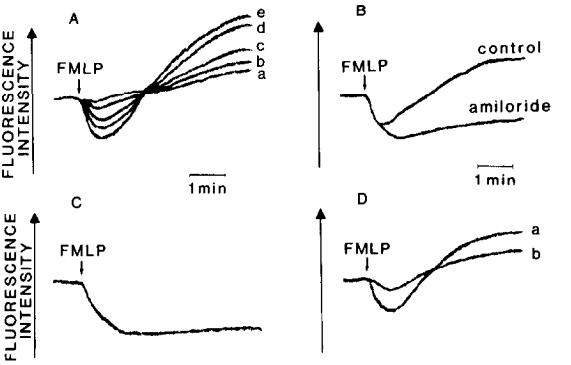

Fig. 1. Effects of FMLP on pHi changes in neutrophils. A) FMLP at various concentrations was added to the cells suspended in NaCl-medium. The final concentration of FMLP was as follows: a) 0.1 nM, b) 1 nM, c) 5 nM, d) 10 nM, e) 100 nM. B) 100 nM FMLP was added to the cells in NaCl-medium which were preincubated with or without 1 mM amiloride. C) 100 nM FMLP was added to the cells in choline-medium. D) 100 nM FMLP was added to the cells in NaCl-medium(a) or in Ca^{2+}-free NaCl-medium 2 minutes after addition of 0.1 mM EGTA(b). The arrows indicate the addition of FMLP. The traces are representative of three similar experiments.

tion lasted for about 30 seconds, the alkalinization started and lasted for about 5 minutes. The dependence of pHi changes on the concentration of FMLP is shown in Fig. 1A. Half maximal activity for the pHi changes by FMLP was obtained at a concentration of 5 nM, which was almost equal to that for stimulation of superoxide generation (data not shown).

The alkalinization phase was completely abolished when the neutrophils were pretreated with amiloride, a potent inhibitor of Na^+/H^+ exchanger, while the acidification phase was not affected (Fig. 1B). Replacement of NaCl with choline chloride essentially abolished the alkalinization phase without affecting the acidification phase (Fig. 1C). The results indicate a possibility that FMLP-induced alkalinization may be mediated by the activation of the Na^+/H^+ exchanger. The observation that FMLP-induced pHi elevation exceeded the resting pHi level may suggest an increased affinity of the exchanger to proton. Grinstein and Furuya reported similar results by using a different fluorescent probe (4).

In the abscence of extracellular free Ca^{2+} and the presence of 0.1 mM EGTA, both acidification and alkalinization phases were markedly decreased (Fig. 1D), indicating that both phases of FMLP-induced pHi changes are dependent on the extracellular Ca^{2+}. This does not agree with the observation of

Sha'afi et al.(3) that only the acidification phase was abolished in Ca^{2+}-free medium without EGTA. Because the intracellular free Ca^{2+} level measured by quin-2 fluorescence was not changed by the treatment of 0.1 mM EGTA for 2 minutes (data not shown), this disagreement may not be due to the difference in the intracellular free Ca^{2+} level but to the difference in the method of pHi measurement or leukocytes used. They measured pHi by estimating the distribution of a labelled weak acid and used rabbit peritoneal leukocytes.

Fig. 2A shows that A-23187 also evoked the biphasic pHi change as FMLP did in a dose dependent manner. In the absence of extracellular Ca^{2+} (Fig. 2D), the pHi changes of both phases were not observed. This observation also supports that Ca^{2+} is required for the induction of the pHi changes. Compared with FMLP, the acidification phase of a few minutes was observed before the alkalinization phase started. Amiloride (Fig. 2B) or the replacement of NaCl with choline (Fig. 2C) abolished only the alkalinization phase, indicating that the alkalinization induced by A-23187 is also mediated by the activation of Na^+/H^+ exchanger.

Fig. 3A shows an inhibitory effect of pertussis toxin on pHi changes induced by FMLP. Pertussis toxin inhibited not only the alkalinization but

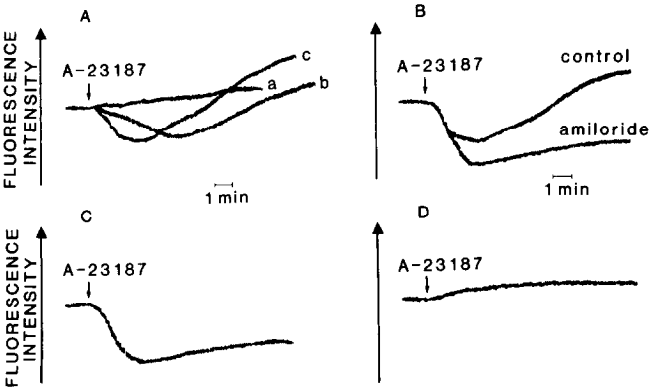

Fig. 2. Effects of A-23187 on pHi changes in neutrophils. A) A-23187 at various concentrations was added to the cells suspended in NaCl-medium. The final concentration of A-23187 was as follows: a) 1 μM b) 5 μM c) 10 μM. B) 10 μM A-23187 was added to the cells in NaCl-medium which were pretreated with or without 1mM amiloride. C) 10 μM A-23187 was added to the cells in choline-medium. D) 10 μM A-23187 was added to the cells in Ca^{2+}-free NaCl-medium. The arrows indicate the addition of A-23187. The traces are representative of three similar experiments.

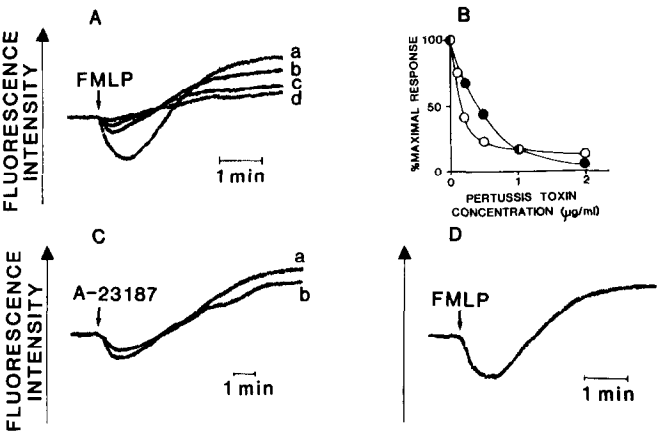

Fig. 3. Effects of pertussis and cholera toxins on pHi changes in neutrophils induced by FMLP or A-23187. A) 100 nM FMLP was added to the cells in NaCl-medium which were pretreated with pertussis toxin (0.1-2.0 μg/ml) at 37°C for 2 hours. a) control cells, b)-d) pertussis toxin-treated cells. The concentrations of pertussis toxin were as follows: b) 0.2 μg/ml c) 0.5 μg/ml d) 1.0 μg/ml. B) Dose-dependence of the inhibition of FMLP-induced pHi changes by pertussis toxin. The magnitude of the FMLP-induced intracellular acidification(o) and alkalinization(●) was measured. C) 10 μM A-23187 was added to the control(a) or pretreated cells with pertussis toxin (1.0 μg/ml) for 2 hours(b) in NaCl-medium. D) 100 nM FMLP was added to the cells in NaCl-medium pretreated with cholera toxin (2.0 μg/ml) for 2 hours. The arrows indicate the addition of FMLP or A-23187. The traces are representative of three similar experiments.

also the acidification phase in a dose-dependent manner. The pHi changes were essentially abolished by pretreatment of the cells with the toxin of 2.0 μg/ml and half maximal inhibitions of the acidification and the alkalinization phases were observed at the concentrations of 200 ng/ml and 400 ng/ml, respectively (Fig. 3B). This inhibitory effect of pertussis toxin may not be due to a decrease in the number or affinity for FMLP receptors, because the toxin affects neither of them (8).

The pHi changes induced by A-23187 were not affected by pretreatment of the cells with pertussis toxin (Fig. 3C). Cholera toxin, which affects a different guanine nucleotide regulatory protein (Ns) from Ni (6, 7), had no effect on pHi changes induced by FMLP (Fig. 3D). Although pertussis toxin is known to enhance cAMP accumulation in response to stimulatory agonists (6, 7), pretreatment of the cells with 2 mM dibutylyl-cAMP did not affect the pHi changes induced by FMLP or A-23187 (data not shown).

A rise in the cytosol Ca^{2+} concentration would be an important factor in the activation of the pHi changes, because inhibition of the FMLP-induced pHi

changes by pertussis toxin may be due to the inhibition of a rise in intracellular Ca^{2+} concentration (10, 11) and A-23187 overcame the inhibitory effect of the toxin. A crucial role of Ca^{2+} has been postulated as the primary trigger for the activation of the Na^+/H^+ exchanger (5, 15). Reactions mediated by Ca^{2+}/calmodulin or Ca^{2+}-activated phospholipid-dependent protein kinase might be involved in the stimulation of the pHi changes. Phorbol 12-myristate 13-acetate which activates the protein kinase directly (16) has been shown to stimulate Na^+/H^+ exchange reaction (17, 18). The molecular basis of the intracellular acidification on stimulation of neutrophils remained to be elucidated.

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