The mechanism underlying the contractile effect of a chemotactic peptide, formyl-Met-Leu-Phe on the guinea-pig Taenia coli

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1The contractile mechanism of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) was investigated in the guinea-pig Taenia coli, by simultaneously monitoring the changes in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and force. 2fMLP induced a significant elevation of [Ca\(^{2+}\)]\(_i\) and force at concentrations higher than 10 nM. The maximal response was obtained at a concentration of higher than 1 μM. 3fMLP (10 μM) augmented the force development by a stepwise increase of the extracellular Ca\(^{2+}\) concentration during 60 mM K\(^+\) depolarization, while it had no effect on the [Ca\(^{2+}\)]\(_i\) elevation, and thus produced a greater force for a given elevation of [Ca\(^{2+}\)]\(_i\) than 60 mM K\(^+\) depolarization. 4The removal of extracellular Ca\(^{2+}\) completely abolished the fMLP-induced contraction. The fMLP-induced [Ca\(^{2+}\)]\(_i\) elevation was inhibited substantially but not completely by 10 μM diltiazem, partly by 10 μM SK&F 96365, and completely by their combination. 5Y27632, a specific inhibitor of rho-kinase, had no significant effect on the fMLP-induced [Ca\(^{2+}\)]\(_i\) elevation and force development. 6Chenodeoxycholic acid, a formyl peptide receptor antagonist, specifically abolished the fMLP-induced contraction but not high K\(^+\)- or carbachol-induced contractions. 7A dual lipooxygenase/cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, a nonselective leukotriene receptor antagonist, and a selective type 1 cysteinyl-containing leukotriene receptor antagonist specifically reduced the fMLP-induced contraction. 8We suggest that the low-affinity-type fMLP receptor and lipooxygenase metabolites of arachidonic acid are involved in the fMLP-induced contraction in the guinea-pig T. coli. This contraction mainly depends on the [Ca\(^{2+}\)]\(_i\) elevation due to Ca\(^{2+}\) influx and the enhancement of Ca\(^{2+}\) sensitivity in the contractile apparatus.

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Abbreviations: [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; [Ca\(^{2+}\)]\(_e\), extracellular Ca\(^{2+}\) concentration; EGTA, ethyleneglycol-bis (β-aminoethylther)-N,N,N′,N′'-tetraacetic acid; fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; PSS, physiological salt solution

Introduction

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), a synthetic analogue of bacterial chemotactic peptide (Schiffmann et al., 1975a, b; Marasco et al., 1984), exerts a potent chemoattractant effect toward neutrophils and monocytes. In addition to this effect, fMLP has been shown to possess spasmodenic properties in smooth muscle tissues including airway, blood vessels, intestinal tract, and renal pelvis (Hamel et al., 1984; Armour et al., 1986; Boukili et al., 1986; Shore et al., 1987; Crowell et al., 1989; Maggi et al., 1992; Minamino et al., 1996). The mechanism for the fMLP-induced contraction appeared to vary with type of smooth muscle tissue. Many studies have suggested an indirect contractile mechanism in the human bronchus, guinea-pig lung parenchymal strips, human coronary artery, and rabbit pulmonary artery (Hamel et al., 1984; Armour et al., 1986; Shore et al., 1987; Crowell et al., 1989; Keitoku et al., 1997). The cyclooxygenase and lipooxygenase metabolites of arachidonic acid, such as thromboxane A\(_2\) and leukotriene C\(_4\), were suggested to mediate the contractile response of fMLP, based on the observation that the inhibitors of cyclooxygenase (Hamel et al., 1984; Shore et al., 1987; Crowell et al., 1989; Keitoku et al., 1997), lipooxygenase (Shore et al., 1987) and thromboxane synthase (Shore et al., 1987), or leukotriene receptor antagonist (Armour et al., 1986; Shore et al., 1987) inhibited the fMLP-induced contraction. The fMLP-activated neutrophils have also been shown to induce endothelium-dependent contraction.
in the human umbilical vein and canine coronary artery (Minamino et al., 1996; Kerr et al., 1998). On the other hand, the direct contractile effect of fMLP has only been suggested in the guinea-pig ileum (Marasco et al., 1982). Furthermore, the cellular mechanism for fMLP-induced contraction remains to be elucidated, especially in terms of intracellular Ca\(^{2+}\) signal transduction.

The cellular effect of fMLP is mediated by formyl peptide receptors. Three genes for humans (formyl peptide receptor (FPR), FPRL1, and FPR2L2) and eight genes for mice (FPR1, FPR-rs1, 2, 3, 4, 5, 6, and 7) have been identified as FPR gene family (Bao et al., 1992; Gao et al., 1998; Wang & Ye, 2002). This receptor family belongs to a superfamily of a seven-transmembrane, G-protein-coupled receptor (Prossnitz & Ye, 1997). Among these receptors, FPR and FPRL1 in humans resemble a variety of cell types including phagocytic leukocytes, lymphocytes, hepatocytes, epithelial cells, neuroblastoma cells, and microvascular endothelial cells (Gronert et al., 1999). FPRL1 is also expressed in a variety of tissues and cells, including neuromuscular, vascular, endocrine, and immune systems (Lacy et al., 1995; McCoy et al., 1995; Sozzani et al., 1995; Becker et al., 1998; Panaro & Mitolo, 1999). FPRL1 is also expressed in a variety of cell types including phagocytic leukocytes, lymphocytes, hepatocytes, epithelial cells, neuroblastoma cells, astrocytoma cells, and microvascular endothelial cells (Gronert et al., 1998; Le et al., 2000). However, the receptor responsible for the fMLP-induced contractile responses remains to be identified.

In the present study, we investigated the mechanism of the fMLP-induced contraction in the guinea-pig Taenia coli, by simultaneously monitoring the cytosolic Ca\(^{2+}\) intensities at 340 (F340) and 380 nm (F380) excitation, and in the absence of any inhibitors) and that obtained at a steady state of contraction induced by 60 mM K\(^{+}\) depolarization were assigned to be 0 and 100%, respectively, in all data analyses.

Methods

Tissue preparation

The experimental protocols were approved by the Animal Care and the Committee of the Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University. Male Hartley guinea-pigs weighing 350-400 g were killed by the intraperitoneal injection of 250 mg kg\(^{-1}\) weight of nembutal and subsequent exsanguination. After the T. coli was isolated, the mucosa and the circumference muscle layer were mechanically removed under a binocular microscope, and then the longitudinal muscle layer was cut into strips (1 mm wide × 3.5 mm long × 0.5 mm thick) as reported previously (Ieiri et al., 2001).

Fura-PE3 loading

The guinea-pig T. coli strips were loaded with the Ca\(^{2+}\) indicator dye, fura-PE3 in the form of acetoxymethyl ester by incubation in oxygenated (a mixture of 95% O\(_2\) and 5% CO\(_2\)) Dulbecco’s modified Eagle’s medium containing 50 μM fura-PE3 acetoxymethyl ester dissolved in dimethylsulfoxide, 5% fetal bovine serum, and 0.02% Pluronic F-127 for 8 h at 37°C (Ieiri et al., 2001). After loading with fura-PE3, the strips were rinsed in normal physiological salt solution (PSS) to remove the dye remaining in the extracellular space, and then were equilibrated in normal PSS for about 1 h before starting the experimental protocols.

Front-surface fluorometry

The changes in the fluorescence intensity of Ca\(^{2+}\)-fura-PE3 complex of the strips of T. coli were monitored with front-surface fluorometry at 37°C, as reported previously (Hirano et al., 1990; Kanaide, 1999; Ieiri et al., 2001). The fluorescence (500 nm) intensities at 340 (F340) and 380 nm (F380) excitation were monitored, and their ratio (F340/F380) was recorded as an indication of [Ca\(^{2+}\)]. The level of the fluorescence ratio obtained at rest (in 5.9 mM K\(^{+}\), 2.5 mM Ca\(^{2+}\)-containing normal PSS, and in the absence of any inhibitors) and that obtained at a steady state of contraction induced by 60 mM K\(^{+}\) depolarization were assigned to be 0 and 100%, respectively, in all data analyses.

Measurement of force development

The strips of T. coli were mounted vertically in a quartz organ bath. One end of the strips was connected to a fixed hook, while the other end was connected to a strain gauge (TB-612-T, Nihon Koden, Japan). The strip was stimulated with 60 mM K\(^{+}\) depolarization every 15 min with a stepwise increase in the resting load until a maximal response was obtained. The optimal resting load thus determined was about 0.7 g. All experimental procedure was performed in the presence of 1 μM tetrodotoxin. The presence of 1 μM tetrodotoxin did not affect the force development induced by 60 mM K\(^{+}\). High K\(^{+}\) depolarization at this concentration reproducibly induces the maximum force development. The level of force obtained at rest (in 5.9 mM K\(^{+}\) and 2.5 mM Ca\(^{2+}\)-containing normal PSS, and in the absence of any inhibitors) and that obtained at a steady state of contraction induced by 60 mM K\(^{+}\) PSS were assigned to be 0 and 100%, respectively, in all data analyses.

Solutions and chemicals

The composition of the normal (5.9 mM K\(^{+}\)) PSS was as follows (in mM): NaCl 123, KCl 4.7, NaHCO\(_3\) 15.5, KH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 1.2, CaCl\(_2\) 2.5, and D-glucose 11.5. The solution containing higher concentrations of K\(^{+}\) was prepared by adding KCl to the normal PSS. A Ca\(^{2+}\)-free solution was prepared by omitting CaCl\(_2\) from normal PSS and adding 0.3 mM ethylenglycrol-bis-[β-aminoethylether]-N,N,N',N'-tetraacetic acid (EGTA). PSS was gassed with a mixture of 5% CO\(_2\) and 95% O\(_2\), with the resulting pH being 7.4. Aspirin, Atropine, BAY u9773, carbamylcholine chloride (carbachol), chenodeoxycholic acid, [des-Arg\(_{10}\)-]HETE 140, HOE 140, famotidine, fMLP, indomethacin, nordihydroguaiaretic acid, phenidone, and pyrilamine were purchased from Sigma (St Louis, MO, U.S.A.). Fura-PE3 acetoxymethyl ester was purchased from Texas Fluorescence Laboratory (Austin, TX, U.S.A.). Dulbecco’s modified Eagle’s medium was purchased...
Data analysis

All data from the simultaneous measurements of \([\text{Ca}^{2+}]\), and force were collected using a computerized data acquisition system (MacLab; Analog Digital instruments, Castle Hill, Australia; Macintosh, Apple Computer, Cupertino, CA, U.S.A.). The representative traces shown were directly printed from the data obtained using this system. All data are the mean±s.e.m. (n = number of the experiments). A strip obtained from one animal was used for each experiment; therefore, the n value indicates the number of animals. A statistical analysis was performed using an unpaired Student’s t-test or a repeated measure analysis of variance. P-values of less than 0.05 were considered to be statistically significant.

Results

fMLP induces sustained elevations of \([\text{Ca}^{2+}]\), and force in the guinea-pig T. coli

The application of 10 \(\mu\text{M}\) fMLP induced a sustained contraction in the guinea-pig T. coli (Figure 1a). fMLP induced a rapid elevation of \([\text{Ca}^{2+}]\), while reaching a peak within 4 min. Thereafter, \([\text{Ca}^{2+}]\), slightly decreased to a sustained level. On the other hand, the force rapidly developed after the application of 10 \(\mu\text{M}\) fMLP, and reached a sustained level within 4 min. It is worth noting that there was a lag time after the application of fMLP until the \([\text{Ca}^{2+}]\), and force started to increase. The lag times for \([\text{Ca}^{2+}]\), and force were 28.8±0.1 and 31.1±0.1 s (n = 9), respectively (Figure 1b). The sustained level of \([\text{Ca}^{2+}]\), and force was maintained for about 15 min and then gradually decreased. A significant elevation of \([\text{Ca}^{2+}]\), and force was observed at the concentrations higher than 0.01 \(\mu\text{M}\), and the EC_{50} values for \([\text{Ca}^{2+}]\), and force were 0.055 and 0.099 \(\mu\text{M}\) (n = 5–9), respectively. On the other hand, 60 \(\mu\text{M}\) K^+ depolarization induced an abrupt elevation of \([\text{Ca}^{2+}]\), and force, without any obvious lag times (Figure 1). The level of \([\text{Ca}^{2+}]\), and the force obtained at rest (in 5.9 \(\text{mM}\) K^+, 2.5 \(\text{mM}\) Ca^2+-containing normal PSS) and that obtained at a steady state of contraction induced by 60 \(\text{mM}\) K^+ were assigned to be 0 and 100%, respectively. A significant elevation of \([\text{Ca}^{2+}]\), and force was obtained with fMLP was significantly (P < 0.05) lower than the force development, thus suggesting that the \text{Ca}^{2+} sensitivity of the contractile apparatus was enhanced by fMLP.

Effect of fMLP on the \([\text{Ca}^{2+}]\); force relationships

To further evaluate the effect of fMLP on the \text{Ca}^{2+} sensitivity of the contractile apparatus of the guinea-pig T. coli, we examined the effect of fMLP on the change in \([\text{Ca}^{2+}]\), and force induced by the stepwise increment of extracellular \text{Ca}^{2+} concentrations ([Ca^{2+}]_o) during the stimulations with 60 \(\text{mM}\) K^+ depolarization. As shown in Figure 2a, after recording the reference response, the strips were exposed to 0.3 \(\text{mM}\) EGTA-containing Ca^{2+}-free PSS for 10 min and to the Ca^{2+}-free media without EGTA for 5 min, and then stimulated with 60 \(\text{mM}\) K^+ depolarization in the Ca^{2+}-free media. The contraction was then induced by increasing [Ca^{2+}]_o from 0 to 2.5 \(\text{mM}\) in a stepwise manner. In the absence of fMLP, the [Ca^{2+}]_o and force increased in a stepwise manner and reached 76.1±7.3 and 89.8±12.4% (n = 5), respectively, at [Ca^{2+}]_o of 2.5 \(\text{mM}\). In order to determine the effect of fMLP on this contraction, 10 \(\mu\text{M}\) fMLP was applied 5 min prior to and during the contraction. As shown in Figure 2b, the addition of 10 \(\mu\text{M}\) fMLP during the 60 \(\text{mM}\) K^+ depolarization in the Ca^{2+}-free PSS induced no elevation of [Ca^{2+}]_o and force, and the
increase in [Ca$^{2+}$], induced by a stepwise increase in [Ca$^{2+}$], in the presence of 10 μM fMLP did not significantly differ from that seen in its absence. However, the development of force was significantly enhanced by the addition of fMLP. The level of [Ca$^{2+}$]$_o$ obtained at each step. The level of [Ca$^{2+}$] and force at rest (5.9 mM K$^+$-PSS) and at the steady state of contraction induced by the first stimulation with 60 mM K$^+$ depolarization was assigned to be 0 and 100%, respectively, 0.3 G, 0.3 mM EGTA. (b) Summary of the changes in [Ca$^{2+}$]$_o$ (left) and force (right) induced by the cumulative application of [Ca$^{2+}$]$_o$ during 60 mM K$^+$ depolarization in the presence and absence of 10 μM fMLP. fMLP was applied 5 min prior to and during the contraction induced by the application of extracellular Ca$^{2+}$. The data are the mean ± s.e.m. (n = 4–5). *Significantly (P < 0.05) different from the values obtained without fMLP. (c) The [Ca$^{2+}$]$_o$–force relation curves reconstructed from the data in (b).

**Figure 2** Effect of fMLP on the contraction induced by the cumulative application of the extracellular Ca$^{2+}$ during 60 mM K$^+$ depolarization in the guinea-pig T. coli. (a) A representative recording showing the changes in [Ca$^{2+}$] and force induced by the stepwise increment of [Ca$^{2+}$]$_o$ during the 60 mM K$^+$ depolarization in the absence of fMLP. The numbers noted by the arrow heads indicate [Ca$^{2+}$]$_o$ obtained at each step. The level of [Ca$^{2+}$] and force at rest (5.9 mM K$^+$–PSS) and at the steady state of contraction induced by the stepwise increment of [Ca$^{2+}$]$_o$ during the 60 mM K$^+$ depolarization was assigned to be 0 and 100%, respectively, 0.3 G, 0.3 mM EGTA.

Negligible contribution of the intracellular Ca$^{2+}$ store to the fMLP-induced contraction

To evaluate the involvement of the Ca$^{2+}$ release from the intracellular store in the fMLP-induced contraction, we applied 10 μM fMLP in the Ca$^{2+}$-free PSS containing 0.3 mM EGTA. When the strip was exposed to Ca$^{2+}$-free PSS containing 0.3 mM EGTA, the [Ca$^{2+}$]$_o$ decreased to $-25.7 ± 2.8\%$ (n = 12) within 3 min, with no change in the resting force. The depolarization with 60 mM K$^+$ induced no increase in either [Ca$^{2+}$]$_o$ or force, thus indicating that [Ca$^{2+}$]$_o$ was nominally ‘0’ (data not shown). In the Ca$^{2+}$-free PSS containing 0.3 mM EGTA, 10 μM fMLP did not induce any increase in [Ca$^{2+}$], and force (Figure 3a). To further examine the role of Ca$^{2+}$ release in the fMLP-induced contraction, we examined the effect of pretreatment with fMLP on the subsequent response to carbachol. We previously reported the carbachol-induced contraction to be dependent on the [Ca$^{2+}$]$_o$ elevation due to Ca$^{2+}$ release in the absence of extracellular Ca$^{2+}$ (Ieiri et al., 2001). As shown in Figure 3b, 10 μM
carbachol induced a small transient elevation of \([\text{Ca}^{2+}]_i\) (from \(-27.2 \pm 4.0 \% \) to \(-14.1 \pm 5.8 \% \), \(n = 6\)) and a large transient development of force (\(162.0 \pm 17.9 \% \), \(n = 6\)) in the absence of \(10 \mu M\) fMLP. The pretreatment with \(10 \mu M\) fMLP had no significant effect on the subsequent changes in \([\text{Ca}^{2+}]_i\) and force induced by \(10 \mu M\) carbachol (Figure 3c). The peak levels of \([\text{Ca}^{2+}]_i\) and force were expressed as a percentage, assigning the values in normal (\(5.9 \mu M \text{K}^+\)) PSS and \(60 \mu M \text{K}^+\) PSS to be 0 and 100\%, respectively. The bottom and top of each column represent the levels of \([\text{Ca}^{2+}]_i\) and force obtained just before and at the maximal elevation after stimulation with carbachol, respectively. The data are the mean±s.e.m. (\(n = 5–6\)). NS, not significantly different (\(P > 0.05\)).

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Effects of diltiazem, SK&F 96365 and Y27632 on the increases in \([\text{Ca}^{2+}]_i\) and force induced by fMLP

To elucidate the mechanism of the sustained phase of the fMLP-induced contraction seen in the presence of extracellular \(Ca^{2+}\), the effects of two types of \(Ca^{2+}\) entry blocker, diltiazem (a L-type \(Ca^{2+}\) channel blocker) and SK&F 96365 (a receptor-operated \(Ca^{2+}\) channel blocker) (Merritt et al., 1990), and a specific inhibitor of rho-kinase, Y27632 (Uehata et al., 1997), on the fMLP-induced contraction were examined. Since parasympathetic innervation plays an important role in the regulation of intestinal contractility, the effects of these inhibitors on the carbachol-induced contraction were also examined, and compared to those seen with the fMLP-induced contraction. In this measurement series, we used fMLP and carbachol at a concentration of \(10 \mu M\) to induce their maximal effect. All inhibitors were applied 10 min before and during contraction induced by fMLP and carbachol, and their effects were evaluated at 15 min after the initiation of contraction (Figures 4b and 5). Pretreatment with \(10 \mu M\) diltiazem, \(10 \mu M\) SK&F 96365, and \(10 \mu M\) Y27632 had no statistically significant effects on the resting levels of \([\text{Ca}^{2+}]_i\) and force. Diltiazem (\(10 \mu M\)) substantially but not completely inhibited the increase in \([\text{Ca}^{2+}]_i\), induced by fMLP, while it almost completely inhibited the force development (Figure 4b). Higher concentrations of diltiazem did not cause any further inhibition (data not shown). On the other hand, SK&F 96365 partially inhibited the increase in \([\text{Ca}^{2+}]_i\), while it had no significant effect on the force development (Figures 4c and 5). The combination of \(10 \mu M\) diltiazem and \(10 \mu M\) SK&F 96365...
completely abolished the fMLP-induced $[\text{Ca}^{2+}]_i$ elevation (Figure 5). The pretreatment with 10 $\mu\text{M}$ Y27632 had no significant effects on the level of $[\text{Ca}^{2+}]_i$ and force seen at 15 min after stimulation with fMLP (Figures 4d and 5).

On the other hand, carbachol induced an abrupt elevation of $[\text{Ca}^{2+}]_i$ and force development with no obvious lag time, followed by a sustained elevation (Figure 4e). It is worth noting that, in addition to the presence of the lag time in response to fMLP, fMLP-induced slower initial increase in $[\text{Ca}^{2+}]_i$, and force than that seen with carbachol (Figures 1a and 4a, e). Diltiazem substantially inhibited the $[\text{Ca}^{2+}]_i$ elevation during the sustained phase, while it almost completely inhibited the force development (Figures 4f and 5). The initial and transient phase of $[\text{Ca}^{2+}]_i$ elevation and force development was also significantly inhibited by diltiazem (Figure 4f). SK&F 96365 significantly inhibited the sustained phase of the carbachol-induced $[\text{Ca}^{2+}]_i$ elevation and force development, while it slightly inhibited the initial phase of...
[Ca^{2+}] increase (Figures 4g and 5). Y27632 had no significant effect on both the initial and sustained elevation of [Ca^{2+}], while it markedly inhibited the sustained level of force (Figures 4h and 5).

**Effects of chenodeoxycholic acid on the fMLP-induced contraction**

In order to determine the involvement of formyl peptide receptor in the fMLP-induced contraction, the effect of a formyl peptide receptor antagonist, chenodeoxycholic acid, was examined. Chenodeoxycholic acid (0–100 μM) was applied 10 min before and during the contraction induced by 10 μM fMLP, 10 μM carbachol, and 60 mM K⁺ depolarization, and its effect was evaluated at the peak of the contraction. As shown in Figure 6, chenodeoxycholic acid inhibited elevation of both [Ca^{2+}] and force induced by fMLP in a concentration-dependent manner, while demonstrating a complete inhibition at 100 μM. The half-maximum inhibitory concentrations (IC₅₀) of chenodeoxycholic acid for fMLP-induced elevations of [Ca^{2+}] and force were 59.4 and 63.3 μM, respectively (n = 4). On the other hand, chenodeoxycholic acid, at any concentrations examined, had no significant effect on the contraction induced by 10 μM carbachol. The elevation of force induced by 60 mM K⁺ was significantly but only slightly inhibited by 100 μM chenodeoxycholic acid. However, 1 mM and higher concentrations of chenodeoxycholic acid completely inhibited the contraction induced by not only fMLP but also carbachol and 60 mM K⁺ (data not shown).

We also examined the effects of other receptor antagonists on the fMLP-induced contraction. The antagonists and the concentrations that we used were 10 μM atropine (muscarinic acetylcholine receptor), 1 μM [des-Arg²⁹]–HOE 140 (bradykinin B₁ receptor), 0.1 μM HOE 140 (bradykinin B₂ receptor), 0.1 μM pyrilamine (histamine H₁ receptor), 1 μM famotidine...
(histamine H₂ receptor), 0.1 µM SR 140333 (tachykinin NK₁ receptor), and 0.1 µM SR 48968 (tachykinin NK₂ receptor). All these antagonists had no significant effect on the fMLP-induced [Ca²⁺] elevation and force development (data not shown). In the guinea-pig T. coli, the contractions induced by carbachol, bradykinin, histamine, and substance P were significantly antagonized by 10 µM atropine, 0.1 µM HOE 140, 0.1 µM pyrilamine, and 0.1 µM SR 140333, respectively (data not shown).

Involvement of lipooxygenase metabolites of arachidonic acid in the fMLP-induced contraction

The possible involvement of lipooxygenase or cyclooxygenase metabolites of arachidonic acid in the fMLP-induced contraction was examined by using inhibitors of lipooxygenase and cyclooxygenase, and leukotriene receptor antagonists as shown in Figure 7. All inhibitors and antagonists were applied 10 min prior and during the contraction induced by 10 µM fMLP, and their effect was examined at the peak of the fMLP-induced contraction. The inhibition of both lipooxygenase and cyclooxygenase by 30 µM phenidone significantly inhibited the fMLP-induced elevation of [Ca²⁺] and force development (Figure 7). The inhibition of lipooxygenase by 1 µM nordihydroguaiaretic acid significantly inhibited the fMLP-induced contractile response. On the other hand, the inhibition of cyclooxygenase by 100 µM aspirin or 10 µM indomethacin had no significant effect on the fMLP-induced force development. Aspirin did not inhibit the fMLP-induced elevation of [Ca²⁺]. Since indomethacin caused an artifact in fluorescence studies, its effect on [Ca²⁺] was not evaluated (data not shown). In line with these observations, a nonselective leukotriene receptor antagonist, ONO-1078 (1 µM), significantly inhibited the fMLP-induced elevation of [Ca²⁺], and force. A selective leukotriene Cys LT₁ antagonist, MK-571 (0.3 µM), also significantly inhibited the fMLP-induced contractile response. An antagonist with a higher affinity toward leukotriene receptor Cys LT₃ than Cys LT₁, BAY u9773 (10 µM), demonstrated no significant effect. A leukotriene B₄ receptor antagonist, U-75302 (1 µM), demonstrated no significant effect. None of the inhibitors or antagonists at the indicated concentrations had any significant effect on the contractions induced by 60 mM K⁺ or 10 µM carbachol, while higher concentrations caused a nonspecific effect on these contractions (data not shown).

Discussion

The contractile effect of fMLP on the smooth muscle has been reported in various tissues including guinea-pig ileum (Marasco et al., 1982), human bronchus (Armour et al., 1986), rabbit pulmonary artery (Crowell et al., 1989), human coronary artery (Keitoku et al., 1997), and guinea-pig renal pelvis (Maggi et al., 1992). We demonstrated that fMLP exerts a contractile effect by activating a formyl peptide receptor in the guinea-pig T. coli. This conclusion is based on the observation that chenodeoxycholic acid, a formyl peptide receptor antagonist, specifically abolished the fMLP-induced contraction in a dose-dependent manner but not the contraction induced by high K⁺ depolarization or carbachol. A receptor similar to that expressed in leukocyte has been suggested to be involved in the fMLP-induced contraction of the guinea-pig ileum (Marasco et al., 1982). However, the molecular identity of the receptor remains unknown. Among the identified receptor genes, human FPR and FPRL1 and mouse FPR1 and FPR-rs2 could serve as a functional receptor for fMLP (Le et al., 2002; Wang & Ye, 2002). fMLP binds to FPR with Kᵣ value in the picomolar to low nanomolar range (high-affinity receptor), while it binds to FPRL1 with Kᵣ value in micromolar range (low-affinity receptor) (Ye et al., 1992; Quehenberger et al., 1993). Chenodeoxycholic acid has been shown to act as a competitive antagonist of both FPR and FPRL1 at concentrations ≤ 150 µM (Chen et al., 2000). In the
present study, the nanomolar to micromolar concentrations of fMLP were required to induce a significant elevation of [Ca\(^{2+}\)], and force development, and chenodeoxycholic acid inhibited fMLP-induced [Ca\(^{2+}\)] elevation and force development at concentrations \(\leq 100 \mu M\). We thus suggest that a low-affinity receptor similar to human FPRL1 is involved in the fMLP-induced contraction in the guinea-pig *T. coli*.

In the rabbit pulmonary artery, human bronchus, and guinea-pig airway, the cyclooxygenase and/or lipoxygenase metabolites of arachidonic acid such as thromboxane A\(_2\) and leukotriene C\(_4\) have been suggested to mediate the fMLP-induced contractile effects (Hamel *et al.*, 1984; Armour *et al.*, 1986; Shore *et al.*, 1987; Crowell *et al.*, 1989; Keitoku *et al.*, 1997). In the present study, lipoxygenase metabolites but not cyclooxygenase metabolites are suggested to make a major contribution to fMLP-induced contraction. The observations with leukotriene receptor antagonists suggest that leukotriene receptor Cys Lt1, but not Cys Lt2 receptor or leukotriene B\(_4\) receptor, plays a major role in such fMLP-induced contraction. Leukotrienes such as D\(_4\) and C\(_4\) species are thus suggested to be major contractile mediators of the fMLP-induced contraction in the guinea-pig *T. coli* (Hæggestrom & Wetterholm, 2002; Izumi *et al.*, 2002; Martel-Pelletier *et al.*, 2003; Mazzetti *et al.*, 2003).

These findings thus suggest that fMLP does not directly evoke contractile responses in the guinea-pig *T. coli*, but lipoxygenase metabolites of arachidonic acid such as leukotrienes mediate the fMLP-induced contraction. The indirect contractile effect of fMLP is consistent with the observation that the [Ca\(^{2+}\)] and force started to increase with a 30-s lag time after stimulation with fMLP. The indirect contractile mechanism has been reported in human airways, guinea-pig lung (Armour *et al.*, 1986; Shore *et al.*, 1987). However, it remains to be elucidated which type of cell is responsible for fMLP-induced production of lipoxygenase metabolites. The strips used in the present study contained smooth muscle cells and interstitial fibroblasts as major cellular components. Leukocytes have also been reported to be responsible for the fMLP-induced, leukotriene-mediated contraction in the human umbilical vein, canine coronary artery, and guinea-pig tracheal smooth muscle (Strek *et al.*, 1993; Minamino *et al.*, 1996; Kerr *et al.*, 1998). Either an autocrine or paracrine mechanism could thus be involved in the fMLP-induced contraction. It still remains to be determined as to which type of cell is the primary cell responding fMLP in the guinea-pig *T. coli*.

In the present study, the contractile mechanism was elucidated in terms of intracellular Ca\(^{2+}\) signal transduction, by simultaneously monitoring the changes in [Ca\(^{2+}\)], and force development and examining the effects of the depletion of the extracellular Ca\(^{2+}\), two Ca\(^{2+}\) entry blockers, and rho-kinase inhibitor on the fMLP-induced contraction. The major findings are as follows: (1) fMLP induced a greater contraction for a given elevation of [Ca\(^{2+}\)], than 60 mM K\(^+\) depolarization. (2) The fMLP-induced [Ca\(^{2+}\)] elevation and force development were completely abolished by the removal of extracellular Ca\(^{2+}\). We thus suggest that fMLP-induced contraction is mainly due to the activation of the Ca\(^{2+}\) influx and enhancement of Ca\(^{2+}\) sensitivity of the contractile apparatus in the guinea-pig *T. coli*.

The intra- and extracellular Ca\(^{2+}\) pools are the major source of the [Ca\(^{2+}\)] elevation induced by receptor stimulation. We suggest that the intracellular Ca\(^{2+}\) store plays a negligible role in the fMLP-induced [Ca\(^{2+}\)] elevation and force development, based on the following observations: first, the removal of the extracellular Ca\(^{2+}\) completely abolished the fMLP-induced contractile response. On the other hand, carbachol did induce a significant elevation of [Ca\(^{2+}\)] and force, thus suggesting the significant involvement of the intracellular Ca\(^{2+}\) pool in the carbachol-induced contraction. Second, the pretreatment with fMLP had no effect on the subsequent carbachol-induced, intracellular Ca\(^{2+}\) pool-dependent [Ca\(^{2+}\)] elevation, and force development. Furthermore, the initial abrupt rising phase of [Ca\(^{2+}\)] and force seen with carbachol-induced contraction was missing in the fMLP-induced contraction. Such an initial phase of [Ca\(^{2+}\)] elevation and force development was considered to be partly due to Ca\(^{2+}\) release from the intracellular pool and partly due to the Ca\(^{2+}\) influx from the extracellular pool, because the initial phase was substantially but not completely inhibited by removing the extracellular Ca\(^{2+}\) or by diltiazem. On the other hand, the Ca\(^{2+}\) influx activated by fMLP is mostly diltiazem sensitive, thus suggesting that the voltage-operated Ca\(^{2+}\) channels plays a major role in the fMLP-induced [Ca\(^{2+}\)] elevation. In the guinea-pig *T. coli*, the elevation of [Ca\(^{2+}\)] induced by elevating the extracellular K\(^+\) reaches the maximal at 40 mM K\(^+\) and higher concentrations (data not shown). The activation of the voltage-dependent Ca\(^{2+}\) influx is thus considered to reach a maximal level at 60 mM K\(^+\). The stimulation with 10 \(\mu M\) fMLP during the 60 mM K\(^+\) -induced contraction induced no further increase in [Ca\(^{2+}\)] (data not shown). This observation thus also supports the involvement of the voltage-dependent Ca\(^{2+}\) influx in the fMLP-induced [Ca\(^{2+}\)] elevation. However, diltiazem did not completely inhibit the fMLP-induced [Ca\(^{2+}\)] elevation, SK&F 96365 partly but significantly inhibited it, and the combination of diltiazem and SK&F 96365 completely abolished the fMLP-induced [Ca\(^{2+}\)] elevation. These findings suggest that the nonvoltage-operated Ca\(^{2+}\) channels are also involved; however, they only play a minor role in the fMLP-induced [Ca\(^{2+}\)] elevation. The major contribution of the Ca\(^{2+}\) influx mainly through the voltage-operated Ca\(^{2+}\) channels and partly through the nonvoltage-operated Ca\(^{2+}\) channels and the negligible contraction of store Ca\(^{2+}\) release to the fMLP-induced contraction is consistent with our previous observations in the leukotriene C\(_{4}\)-mediated contraction (Ieiri *et al.*, 2001), thus supporting the involvement of leukotrienes in the fMLP-induced contraction.

It is well established that receptor stimulation induces contraction not only by increasing [Ca\(^{2+}\)], but also enhancing the Ca\(^{2+}\) sensitivity of the smooth muscle contractile apparatus (Somlyo *et al.*, 1999). Rho-kinase has been recently shown to play an important role in the enhancement of the Ca\(^{2+}\) sensitivity by inhibiting the dephosphorylation of myosin by or by directly activating myosin phosphorylation (Amano *et al.*, 1996; Kimura *et al.*, 1996). However, the observation that a rho-kinase inhibitor, Y27632, had no significant effect on the fMLP-induced force development excludes a major contribution of rho-kinase to the fMLP-induced Ca\(^{2+}\)-sensitizing effect. Many other kinases, including Zip or Zip-like kinase, integrin-linked kinase, and myotonic dystrophy protein kinase, have been shown to induce the Ca\(^{2+}\)-independent phosphorylation of myosin light chain or inhibition of myosin phosphatase, and thus suggested to be involved in the enhancement of Ca\(^{2+}\) sensitization (Hirano *et al.*, 2003). It is thus possible that fMLP activated the kinases...
other than rho-kinase and thereby enhanced the Ca\(^{2+}\) sensitivity. However, this possibility still remains to be confirmed. On the other hand, we reported that the myofilament Ca\(^{2+}\) sensitivity was not enhanced in the leukotriene C\(_4\)-induced contraction (Ieiri et al., 2001), which is inconsistent with the observation in the present study that fMLP increased the myofilament Ca\(^{2+}\) sensitivity. Leukotriene C\(_4\) could not account for all contractile mediators involved in the fMLP-induced contraction.

Since fMLP is a synthetic analogue of the bacterial chemotactic peptide, the observed contractile effect of fMLP may be linked to the contractile abnormality associated with the inflammatory responses to a bacterial infection of the intestine. On the other hand, chenodeoxycholic acid is a bile product, which could serve as an endogenous fMLP receptor antagonist, and thus help to suppress these functional abnormalities related to the fMLP-induced contractile effect in infectious bowel disease.

In conclusion, we have demonstrated the contractile effect of fMLP in the guinea-pig T. coli. Our findings suggest that this effect is mediated by a low-affinity formyl peptide receptor similar to human FPR1 or mouse FPR1-rs2. However, fMLP is not suggested to induce directly a contractile response, but lipoxigenase metabolites and leukotriene receptor Cys L\(_1\) are suggested to mediate directly the contractile responses. The Ca\(^{2+}\) influx, mainly through the voltage-operated Ca\(^{2+}\) channels and partly through the nonvoltage-operated Ca\(^{2+}\) channels, and the enhancement of the Ca\(^{2+}\) sensitivity of the contractile apparatus are the main intracellular mechanisms for fMLP-induced contraction. The Ca\(^{2+}\) mobilization from the intracellular store sites was suggested to play a negligible role in fMLP-induced contraction, while rho-kinase may not play any major role in it at all. The contractile effect mediated by the formyl peptide receptor expressed in the intestinal smooth muscle may therefore play an important role in the pathophysiology related to the acute inflammatory response to bacterial infection.

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References


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