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MOTILIN INDUCES THE ENDOTHELIUM-DEPENDENT RELAXATION OF SMOOTH MUSCLE AND THE ELEVATION OF CYTOSOLIC CALCIUM IN ENDOTHELIAL CELLS *IN SITU*

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Using front-surface fluorometry with fura-2 and porcine aortic valvular strips, we investigated the effect of motilin, a gastrointestinal peptide, on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of endothelial cells *in situ*. Motilin induced a biphasic elevation of $[Ca^{2+}]_i$ of the endothelial cells on the porcine aortic valvular strips. To investigate the physiological role of these Ca^{2+} transients in the endothelial cells, we determined the effect of motilin on $[Ca^{2+}]_i$ as well as the tension of the smooth muscles in the porcine coronary strips with an intact endothelium. Motilin decreased $[Ca^{2+}]_i$ and the tension of the coronary smooth muscle precontracted by U46619, a thromboxane A2 analogue, in an endothelium-dependent manner. In the presence of indomethacin (a cycloxygenase inhibitor), motilin induced an endothelium-dependent relaxation of the coronary strips which was partially inhibited by N^{ω}-nitro-L-arginine (L-NNA; a NO synthase inhibitor). These results thus indicate that motilin induces Ca^{2+} transients of the endothelial cells while it also induces vasorelaxation, which may be mediated by both L-NNA sensitive and resistant factors that are derived from the endothelium.

Motilin is a 22 amino acid polypeptide originally isolated from the porcine gastrointestinal tract (1) which is a potent stimulator of contractility of the gastrointestinal muscle both *in vivo* and *in vitro* (2-5). It has been reported that motilin directly activates the intestinal smooth muscle cells both in man and rabbits (5-7). The isolated guinea pig stomach smooth muscle cells also responded to motilin (8). Besides the gastrointestinal tract, Eimerl *et al.*(9) have also suggested that motilin

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ABBREVIATIONS : $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; L-NNA, N^{ω}-nitro-L-arginine; EDRF, endothelium-derived relaxing factor; EDHF, endothelium-derived hyperpolarizing factor; PGI_2, prostacyclin; Fura-2/AM, acetoxymethyl ester form of fura-2; PSS, physiological salt solution; ATP, adenosine triphosphate; NO, nitric oxide.

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acts directly on the peripheral vascular smooth muscle cells in the pithed rat model. The present study is the first to describe the effect of motilin on endothelial cells.

It is generally accepted that the changes in $[Ca^{2+}]_i$ of the endothelial cells play an essential role in the regulation of the production and the release of vasorelaxing substances (10-12) such as endothelium-derived relaxing factor (EDRF) (13), endothelium-derived hyperpolarizing factor (EDHF) (14) and prostacyclin (PGI_2) (15). In this study, we determined the effect of motilin on $[Ca^{2+}]_i$ of endothelial cells on the aortic side of the porcine aortic valve, using fura-2 front surface fluorometry (16). To determine the effect of motilin on the function of endothelial cells, we measured $[Ca^{2+}]_i$ and the tension of smooth muscles in porcine coronary arterial strips with an intact endothelium precontracted by U46619. We thus found that motilin induces $[Ca^{2+}]_i$ transients in endothelial cells while it also causes endothelium-dependent vasorelaxation.

MATERIALS AND METHODS

The measurement of $[Ca^{2+}]_i$ of the endothelial cells in situ

The method to measure the changes in $[Ca^{2+}]_i$ of the endothelial cells *in situ* has been described elsewhere in detail (16). In brief, the valvular strips (3mm×5 mm), dissected from the porcine aortic root, were loaded with Ca^{2+} indicator dye, fura-2, by incubating in oxygenated (5% CO_2, 95% O_2) Dulbecco's modified Eagles medium containing 50 μM fura-2/AM and 5% fetal bovine serum and 1 mM probenecid for 90 minutes at 37°C. Each strip was then mounted vertically in a quartz organ bath and any changes in the $[Ca^{2+}]_i$ of the endothelial cells on the surface of the aortic side were monitored using front surface fluorometer (CAM-OF-1; Japan Spectroscopic Co., Tokyo, Japan). The measurements were carried out at 25°C to prevent any leakage of fluorescent dye from the cells. The fluorescence intensities at 340 nm (F340) excitation and at 380 nm (F380) excitation and their ratios (R=F340/F380) were monitored at 500 nm emission. The response to 10 μM ATP was registered 15 min prior to each experiment, and the resting levels and the peak level of the fluorescence ratio were designated to be 0% and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ were calculated using the method described by Grynkiewicz *et al.* (17) with minor modifications. The $[Ca^{2+}]_i$ levels at 0% and 100% were determined in separate experiments, and they were 65.3 ± 7.5 nM and 185.9 ± 20.8 nM, respectively (n=10).

The measurement of $[Ca^{2+}]_i$ and tension of smooth muscle in the coronary arterial strips

The simultaneous measurements of $[Ca^{2+}]_i$ and the tension of the proximal part of porcine coronary arterial smooth muscle cells either with or without an endothelium were performed as described earlier in detail (18). In brief, both the endothelium-intact and endothelium-removed vascular strips were loaded with fura-2, by incubation in oxygenated Dullbecco's modified Eagles medium containing 50 μM fura-2/AM and 2.5 % fetal bovine serum for 3-4 h at 37°C. The strips were then washed by normal PSS three times and transferred to oxygenated warm PSS (37°C) for at least 60 min to remove any remaining dye in the extracellular spaces and to equilibrate the strips before starting the measurements. The fura-2 loaded strips were mounted vertically in a quartz organ bath as the luminal side of the strip faced the front of the bath. Tension development was measured using a strain gauge (TB-612T, Nihon Koden, Japan). The strips were stimulated by 118 mM K^+ depolarization four or five times repeatedly before each experiment and then the resting tension was adjusted to 200-300 mg. The developed tension was expressed as a percentage of the control 118 mM K^+ depolarization-induced tension development measured at 10 min after application (steady-state) and the values in normal PSS (5.9 mM K^+) were designated to be 0 % while the values in 118 mM K^+ depolarization were designated to be 100 %. Changes in the fluorescence intensity of the fura-2/Ca^{2+} complex were monitored with a front-surface fluorometer as described above. The ratio of the fluorescence intensities (R=F340/F380) was expressed as a percentage, designating the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0 % and 100 %,

respectively. Under the present experimental conditions, the fura-2 signals were observed to come exclusively from the smooth muscle cells of arterial strips (18). The $[Ca^{2+}]_i$ levels at rest (0 %) and during 118K^+ depolarization (100 %) were 108 ± 27 nM and 715 ± 103 nM, respectively (n=5).

Chemicals and solutions

Motilin (porcine) and sodium salt of ATP were obtained from the Peptide Institute (Osaka, Japan) and Boehringer Mannheim GmbH (Germany), respectively. Fura-2/AM was purchased from Dojindo (Kumamoto, Japan). Probenecid was obtained from SIGMA (St Louis, MO, USA). U46619, L-NNA and indomethacin were purchased from the Aldrich Chemical Co. (USA), Calbiochem Co (USA) and WAKO (Osaka, Japan), respectively. All other chemicals were obtained from Katayama Chemical (Osaka, Japan). The composition of the normal PSS was (in mM); 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose. All solutions were gassed with a mixture of 5 % CO_2 and 95 % O_2. The solution was maintained at 37°C and 10 μM indomethacin was added in the experiment with coronary arterial strips. In contrast, the solution was maintained at 25°C in the experiments using valvular strips.

Data analysis

The values are expressed as the means ± standard error. Student's *t*-test was used to determine statistical significance. $P<0.05$ was considered to be significant. All data were collected by a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, Macintosh; Apple Computer, U.S.A.). The collected data were directly printed out from the computer to a laser printer (LaserWriter II NTX-J, Apple Computer, U.S.A.) in order to make the figures included in this report.

RESULTS

Figure 1A, B and C show the representative recordings of the effect of motilin (1-5 μM) on $[Ca^{2+}]_i$ of the fura-2 loaded endothelial cells on the valvular strips in the presence of 1.25 mM extracellular Ca^{2+}. Motilin (> 3 μM) induced a biphasic elevation of $[Ca^{2+}]_i$ which consisted of a large and transient phase and reached maximal peak levels by 1.5 min after stimulation, followed by a lower and more sustained phase that was attained within the next 10 min. As shown in Fig. 1D, the peak response and sustained level were both concentration-dependent (1-10 μM). The maximal response was observed at a concentration higher than 5 μM. The amplitude of the peak response level and the sustained level obtained by 5 μM motilin were 60.6 ± 4.9 % and 10.0 ± 2.4 % (n=4) of 10 μM ATP-induced elevation, respectively.

Since $[Ca^{2+}]_i$ transient in endothelial cells usually indicates the production and release of vasorelaxing factors, in the next step, we investigated the vasorelaxing effect of motilin on porcine proximal coronary arterial strips with an intact endothelium. To eliminate the effect of PGI_2, we thus performed the following experiments in the presence of 10 μM indomethacin. When 100 nM U46619 was applied to the porcine coronary arterial strips either with or without an endothelium, $[Ca^{2+}]_i$ and the tension rapidly increased and reached steady state levels within 10 min and both levels were thereafter maintained for at least 60 min (data not shown). As shown in Fig. 2A, when 3 μM motilin was applied to the porcine coronary arterial strips without an endothelium during contraction induced by U46619, no change of $[Ca^{2+}]_i$ and the tension were observed. In the strips with an intact endothelium, 3 μM motilin elicited a rapid decrease in $[Ca^{2+}]_i$ and tension ; the maximum reduction of $[Ca^{2+}]_i$ (17.1 ± 1.0 %) and tension (9.5 ± 0.9 %) were observed at 0.9 min

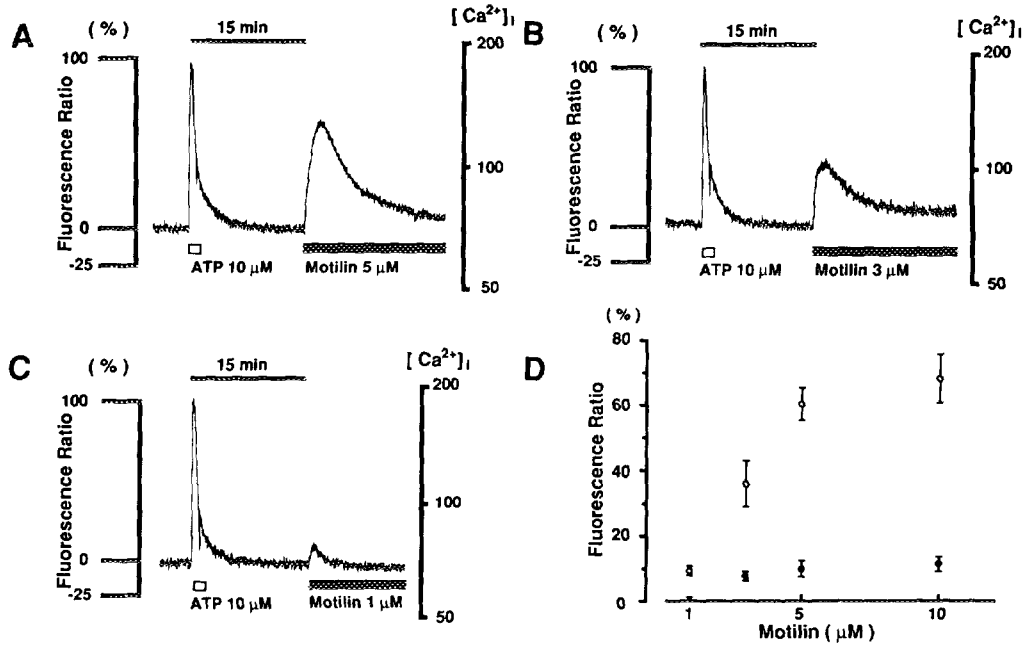

Figure 1.

Representative recordings showing the effect of 1 μM(A), 3 μM(B) and 5μM(C) motilin on $[Ca^{2+}]_i$ in endothelial cells on the porcine aortic valve in normal PSS (1.25 mM extracellular Ca^{2+}). Prior to each experiment, the response to 10 μM ATP for 1 min was recorded as a control. Figure 1D shows the concentration-response curve for the motilin-induced increase in $[Ca^{2+}]_i$. Open and closed circles show the magnitude of the initial transient and the sustained increases in $[Ca^{2+}]_i$, respectively. The fluorescence ratio was expressed in percent (%), assuming the resting level and the peak response induced by 10 μM ATP to be 0% and 100%, respectively. Each point indicates the means ± s.e.(n=3-5).

and 1.5 min after the application of motilin, respectively (n=4), and thereafter $[Ca^{2+}]_i$ and tension gradually returned to levels similar to those observed in the strips without an endothelium at 10 min and 20 min, respectively (Fig. 2B). To determine the extent of the contribution of EDRF to motilin-induced endothelium-dependent relaxation, the strips with an intact endothelium were pretreated with a competitive inhibitor of NO synthase, L-NNA. When the strips with an intact endothelium were pretreated with 100 μM L-NNA (the maximal effect) for 40 min, 3 μM motilin induced a rapid decrease in $[Ca^{2+}]_i$ and tension ; the maximum reduction of $[Ca^{2+}]_i$ (30.7 ± 2.2 %) and tension (43.0 ± 3.9 %) were observed at 0.7 min and 0.9 min after stimulation, respectively (n=4), and $[Ca^{2+}]_i$ and the tension then returned to the levels similar to those observed without an endothelium at 5 min and 10 min, respectively (Fig. 2C).

Figure 3 graphically summarizes the results of the time courses obtained from 4 independent experiments done in a similar manner as that in Figs. 2A, B and C. These graphs clearly show that the extent and duration of the decrease in $[Ca^{2+}]_i$ and tension induced by motilin in the presence of L-NNA were much smaller than that observed in the absence of L-NNA. L-

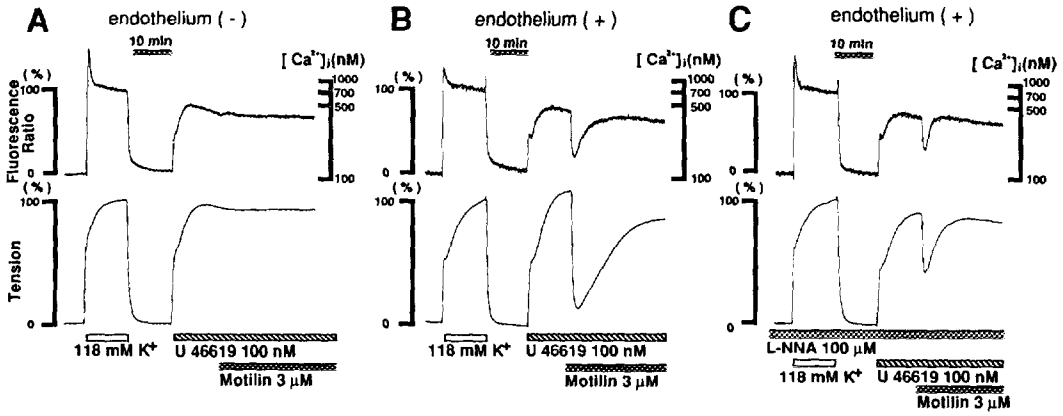

Figure 2.

Representative recordings showing the effects of 3 μM motilin on $[Ca^{2+}]_i$ and the tension of smooth muscles in the porcine coronary arterial strips either without (A) or with (B) an endothelium during contraction induced by 100 nM U46619 and the effect of 100 μM L-NNA on the motilin-induced endothelium-dependent relaxation (C). Prior to each experiment, the response to 118 mM K^+-depolarization was recorded. All experiments were performed in the presence of 10 μM indomethacin. The fluorescence ratio was expressed in percent (%), while assuming the resting level and the steady state level during 118 mM K^+-depolarization to be 0 % and 100 %, respectively.

NNA, even at higher concentrations, therefore only partially inhibited the reduction of $[Ca^{2+}]_i$ and tension induced by motilin.

DISCUSSION

In this study, we demonstrated that motilin induces a biphasic elevation of $[Ca^{2+}]_i$ in endothelial cells *in situ*. The biphasic nature of the elevation of $[Ca^{2+}]_i$ induced by motilin was similar to that of other receptor-operated agonists such as endothelin-1, bradykinin, thrombin and substance P in endothelial cells (19-22). It is well accepted that the initial transient increase in $[Ca^{2+}]_i$ is thought to be due to the intracellular Ca^{2+} release from stores and the steady state is due to the Ca^{2+} influx from the extracellular spaces (19, 23). In the absence of extracellular Ca^{2+}, the steady state induced by motilin was abolished (data not shown). Thus, it is also possible that the motilin-induced biphasic elevation of $[Ca^{2+}]_i$ in endothelial cells *in situ* is due to both the intracellular Ca^{2+} release from the stores as well as the Ca^{2+} influx from the extracellular spaces.

It has been reported that receptors specific to motilin are present on the smooth muscle cells of the guinea pig stomach (8), and the rabbit and human intestine (24, 25) and motilin acts directly on these smooth muscle cells. Our present results tend to indicate that the vascular endothelial cells have motilin receptors. However it should be noted that motilin might activate endothelial cells via receptors other than motilin receptors, because the concentrations needed to activate endothelial

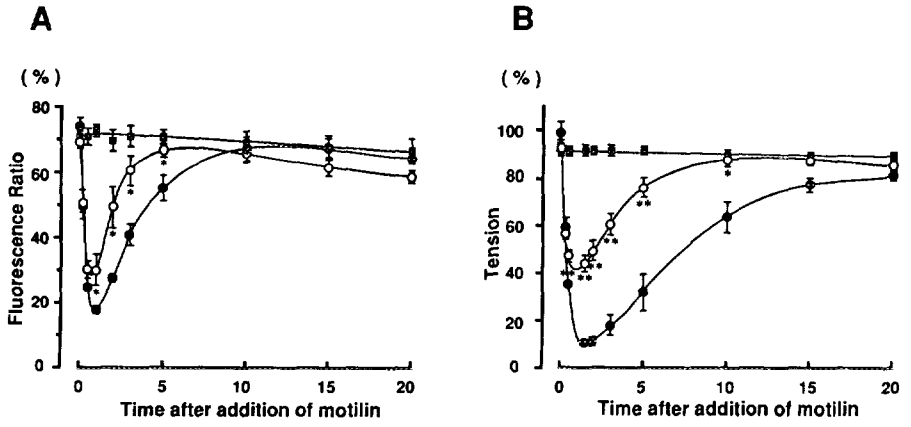

Figure 3.
The time courses of changes in $[Ca^{2+}]_i$ (A) and tension (B) induced by 3 μM motilin in the porcine coronary artery either without (■) or with an endothelium in the presence (○) or absence (●) of 100 nM L-NNA. All experiments were performed in the presence of 10 μM indomethacin. The ordinate indicates the percent of the fluorescence ratio (A) and tension (B). The abscissa shows the time after the addition of motilin. The data were analyzed by Student's *t*-test (n=4). * , $p< 0.05$; ** , $P< 0.001$ (compared with the value in the absence of L-NNA(●) at each panel)

cells in the present study are rather high (μM order), compared with the reported values for gastrointestinal smooth muscle cells (nM order) (7, 8).

We have demonstrated that motilin induces an endothelium-dependent decrease in both $[Ca^{2+}]_i$ and the tension of smooth muscle cells from proximal coronary arterial strips precontracted with U46619 in the presence of indomethacin, and that this decrease is partially inhibited by treatment with L-NNA. Vanhoutte (26) recently reported that endothelium-dependent relaxations, which are not prevented by inhibitors of the L-arginine NO pathway (nitro-L-arginine resistant relaxation) in the presence of indomethacin, are considered to be caused primarily by the effect of EDHF. In the porcine coronary artery, it has been reported that several receptor-operated agonists such as bradykinin, thrombin and substance P induce endothelium-dependent relaxation which is partially mediated by EDHF (12, 27). Therefore, it seems likely that motilin also produces and releases not only L-NNA sensitive (EDRF) but also resistant (EDHF) vasorelaxing factors from the porcine coronary arterial endothelial cells.

The analysis of the time course of the motilin-induced endothelium-dependent decrease in $[Ca^{2+}]_i$ and tension in the absence of L-NNA indicated that it was composed of a rapid decrease and a subsequent relatively sustained decrease in $[Ca^{2+}]_i$ and tension, which then gradually returns to prestimulation levels, in the porcine coronary artery precontracted by U46619 (Figs. 2B and 3). L-NNA inhibited the amplitude of both the rapid and subsequent decrease in $[Ca^{2+}]_i$ and tension (Fig. 3). Although it is still only a rough estimation, it can be suggested that an L-NNA resistant decrease in $[Ca^{2+}]_i$ and tension might contribute to more than 50 % of the reduction of $[Ca^{2+}]_i$ and tension in the rapid phase induced by motilin. This finding is consistent with previous reports that EDHF

initiates the endothelium-dependent relaxation observed in guinea-pig basilar arteries stimulated by acetylcholine (28) and in pig carotid arteries stimulated by substance P (29).

In summary, this is the first report to demonstrate that motilin acts on the endothelial cells to elevate $[Ca^{2+}]_i$ and causes an endothelium-dependent relaxation which is mediated by L-NNA sensitive (EDRF) and resistant vasorelaxing substances (most likely EDHF). Although the physiological role of motilin on the regulation of vascular tone and blood flow is still unknown, it should be noted that motilin has a vasorelaxing effect on the proximal part of the coronary artery with an intact endothelium.

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