POSTSYNAPTIC ENHANCEMENT BY MOTILIN OF MUSCARINIC RECEPTOR CATION CURRENTS IN DUODENAL SMOOTH MUSCLE

KAZUNORI YAMADA, HIROE YANAGIDA, YUSHI ITO, AND RYUJI INOUE
Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan

Postsynaptic enhancement by motilin of muscarinic receptor cation currents in duodenal smooth muscle. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G487–G492, 1998.—We have investigated a potential role of motilin in amplifying the postsynaptic muscarinic responses in the rabbit duodenal smooth muscle cells, using the whole cell variant of patch-clamp technique. Stimulation of motilin receptors by exogenously applied motilin (1 nM) resulted in a large increase in carbocyclic (CCh)-induced atropine-sensitive cation current (I_cation) at threshold concentrations of CCh (0.3–1 µM) at 30°C. This potentiation was abolished in the presence of a specific blocker of motilin receptor (GM109) and was attenuated with increased concentrations of either motilin or CCh, being virtually absent with maximally effective concentrations of these agonists. Motilin failed to potentiate I_CCh when the ambient temperature was reduced to 20°C or if the cation current had been directly activated by internal perfusion with guanosine 5’-O-(3-thiotriphosphate) (50 µM) bypassing the muscarinic receptor. These results suggest that some biochemical processes, such as enzymatic reactions, might be involved in the motilin-induced potentiation and that its site of action might be the muscarinic receptor and/or associated G proteins.

Thus the in vitro results rather favor a direct action of motilin via the postsynaptic motilin receptors on gut smooth muscle. This possibility has recently been confirmed in patch-clamp experiments using single cells dissociated from the rabbit duodenal smooth muscle (29). In this study it has been demonstrated that in addition to causing Ca^2+ release from internal stores and depressing the voltage-dependent Ca^2+ currents, motilin is capable of activating monovalent cation-selective voltage-independent, and Ca^2+-independent channels (29), the properties of which are largely different from those of voltage-dependent divalent cation-permeable cation channels that are linked to the muscarinic receptor via a pertussis toxin-sensitive G protein and are ubiquitously found in the whole gut (muscarinic cation channels; 2, 5, 12, 17).

In the present study we have attempted to reconcile these apparently discrepant results. We have used single dissociated cells from the rabbit duodenal smooth muscle combined with the patch-clamp technique, which helps us to eliminate possible contamination or interactions with nonmuscle factors. As the result of this work, we have found that at least one of the excitatory effects of the cholinergic system on rabbit duodenal smooth muscle, activation of muscarinic cation channels, can be effectively amplified by a preceding stimulation of motilin receptors on the myocyte.

METHODS

Materials and cell dispersion. Albino rabbits of either sex, weighing 1.5–2 kg (Nihon White), were exsanguinated under anesthesia with intravenous pentobarbital. A cylindrical segment of duodenum (about 5 cm from the pylorus) was excised. A plastic pole (about 1 cm in diameter and 10 cm in length) was inserted through the lumen of the cylindrical segment, the ends of which were fixed tightly on the pole using thin silk threads. The segment of duodenum was successively incubated at 35°C in Ca^2+-free physiological salt solution (for composition, see below) for 10–20 min until the whole segment became fully relaxed and then in Ca^2+-free physiological salt solution containing 2 mg/ml collagenase (type I, 250 U/mg) at 35°C for 20–25 min. The digested segment was cut open longitudinally and stored in a refrigerator in 0.5 mM CaCl_2-containing Krebs solution supplemented with 1 mg/ml fat-free bovine serum albumin and 1 mg/ml soybean trypsin inhibitor. Single cells were dispersed just before use (within 6 h after enzymatic digestion), by gently triturating, with a blunt-tipped Pasteur pipette, minced pieces of digested longitudinal muscle that had mechanically been peeled off from the mucosa with fine forceps. The recording system used for the patch-clamp experiments was essentially the same as described previously (29). Briefly, to generate voltage pulse or ramp commands, or to amplify the current signal sampled from the clamped cells, an Axopatch 1-C amplifier (Axon Instruments) was used in conjunction with an analog-to-digital, digital-to-analog converter (MacLab/4; AD Instru-

MOTILIN HAS BEEN IMPLICATED as a gut peptide hormone in initiating an intermittent migrating motor complex in the fasting gut (14, 27). This has been supported by the observation that the plasma motilin levels fluctuate in synchrony with the interdigestive migrating motor complex, but stay depressed during a postprandial period, and that intravenous administration of motilin or CCh when the ambient temperature was reduced to 20°C or if the cation current had been directly activated by internal perfusion with guanosine 5’-O-(3-thiotriphosphate) (50 µM) bypassing the muscarinic receptor. These results suggest that some biochemical processes, such as enzymatic reactions, might be involved in the motilin-induced potentiation and that its site of action might be the muscarinic receptor and/or associated G proteins.

In contrast, as well recognized in in vitro experiments, anticholinergic agents or the axonal conduction blocker (tetrodotoxin) has been found to exert little effect on the contraction of isolated muscle strips elicited by exogenously applied motilin (1, 25, 26, 29).
ments, New South Wales, Australia), which was run under the software Chart v.3. Cell capacitance and series resistance (<15 MΩ) were not compensated. To record long-lasting events, the data were digitized and stored on videotape after pulse code modulation (PCM-501ES, Sony, Tokyo, Japan). For illustration and data analysis, a Macintosh computer (Performa 575) and its standard attached softwares (Microsoft Excel v.4.0; KleidaGraph v.3.04; MacDraw Pro v.1.5) were used. To minimize errors arising from the noisy fluctuating nature of carbachol (CCh)- or motilin-induced currents when determining the current amplitude, the current traces were averaged over a period of at least 2 s before and after the application of agonists.

Solutions. We had confirmed in preliminary experiments that CCh- and motilin-induced inward currents in rabbit duodenal smooth muscle are mainly cationic, as has been reported for other parts of the intestine (2, 12, 17). Thus, to facilitate more selective recording of a cationic current component, high Cs⁺, low Cl⁻ solution was loaded into the cell (for composition, see below), and the membrane was clamped close to the predicted equilibrium potential of Cl⁻ (~−45 mV). Liquid junction potentials arising at the interface between the bathing and internal solutions were measured as described elsewhere (ca. 6 mV; Ref. 28), and corrected a posteriori. The temperature of the bathing solution was kept at 30–31°C (higher temperatures resulted in progressive cell deterioration), except for the experiments shown in Fig. 6. The composition of modified Krebs solution was (in mM) 137 Na⁺, 5.5 K⁺, 1.2 Mg²⁺, 2 Ca²⁺, 132.2 Cl⁻, 15.5 HCO₃⁻, 1.1 H₂PO₄⁻, and 11.9 glucose, continuously aerated with 95% O₂ and CO₂. The composition of Ca²⁺-free cell dispersing solution was (in mM) 140 Na⁺, 5 K⁺, 1.2 Mg²⁺, 147.4 Cl⁻, 11.9 glucose, and 10 N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES), adjusted at pH 7.35–7.4 with tris(hydroxymethyl)aminomethane (Tris) base. The composition of high Cs⁺, low Cl⁻ internal solution was (in mM) 130 Cs⁺, 2 Mg²⁺, 20 Cl⁻, 110 aspartate, 2 SO₄²⁻, 2 Na₂ATP, 5 creatine phosphate (Tris salt), 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4 Ca²⁺, and 10 HEPES, titrated to 7.2 with Tris base.

Chemicals. HEPES and EGTA was purchased from Dojin (Kumamoto, Japan), CCh and porcine motilin from Sigma, and GM109 was a kind gift from Chugai Pharmaceutical.

Statistics. All data are expressed as means ± SE, and statistical significance was evaluated by paired or unpaired t-test with criteria given in each figure.

RESULTS

Figure 1A demonstrates examples of inward currents induced at ~50 mV, in response to CCh added to the bath (1 and 10 µM). Activation of the inward currents, which was strongly attenuated after 4–6 h of pretreatment with pertussis toxin (1 µg/ml, 36°C; data not shown), occurred in a dose-dependent manner with a threshold as low as 0.3 µM (Fig. 1B). Empirical fitting of this dose dependence with a Hill-type equation indicated that the half-maximal activation of the currents occurs at 9 µM with a cooperative coefficient of about 1 (Fig. 1B). These results, in addition to other biophysical features of the currents such as a U-shape, voltage dependence, and reversal potential close to 0 mV under the conditions in which K⁺ and Cl⁻ currents are suppressed (inset in Fig. 2; for ionic conditions see METHODS), strongly suggest that the channel(s) underlying these inward currents may be related to the pertussis toxin-sensitive G protein-coupled, voltage-dependent cation channel family that includes the muscarinic receptor-operated channel, which is the main regulator of membrane excitability in the mammalian gut smooth muscle (2, 5, 11, 17, 18, 23).

Stimulation of motilin receptor (0.1–100 nM), preceding the application of CCh, which itself induces a monovalent cation-selective, voltage-independent current (I_CCh; also see the inset in Fig. 2 and Ref. 29), caused a pronounced enhancement of inward current induced in response to CCh (I_CCh). In the example shown in Fig. 3A, a more than fivefold increase in the amplitude of I_CCh was achieved after the application of motilin (i.e., the inward component superimposed on I_CCh). The enhancing effect of motilin on I_CCh was, however, not consistently observed in all cells tested. Of 86 cells tested, about one-half (41 cells) exhibited clear enhancement; the other cells showed no increase or even a slight decrease. This apparent inconsistency...
could not be accounted for by contamination with nonmuscle cells such as neurons, because almost all cells yielded by our dispersion procedure had the spindle-shaped appearance typical of smooth muscle cells and contracted rapidly to acetylcholine. Variable cell surface damage during the course of enzymatic digestion and time-dependent rundown of I_CCh due to internal dialysis might contribute to the diversity of the results or differing distributions of motilin or muscarinic receptors on the rabbit duodenal smooth muscle.

The enhanced I_CCh seen when superimposed on I_Motr is likely to be a genuine CCh current, because atropine (1 µM) selectively abolished the current component induced by CCh without affecting I_Motr, and conversely GM109, a specific antagonist of motilin (26), antagonized the increasing effect of motilin on I_CCh (Fig. 3, B and C). Furthermore, the enhanced current maintained its characteristic U-shaped current-voltage relationship in the inward portion (Fig. 4C; Refs. 2, 5, 16, 18), whereas that of I_Motr is nearly ohmic in the corresponding portion of membrane potential (compare with Fig. 2), as has already been reported (29). These results strongly suggest that the “enhanced I_CCh” is the result of selective potentiation of the muscarinic receptor-activated cation current.

Potentiation of I_CCh by motilin outlasted the washout of motilin (Fig. 4A), but the extent of potentiation appeared to be paralleled by the magnitude of the underlying I_Motr. As I_Motr declined, the extent of potentiation of I_CCh gradually faded. This might reflect a slow dissociation of motilin from its receptor and/or switch-off of the cellular signaling pathways initiated by motilin-receptor activation. We did not pursue the details of this phenomenon, but no essential difference was found in the extent of potentiation immediately after termination of motilin application (3–10 min) and in the continued presence of motilin. Thus both data were included in the evaluation.

The enhancing effect of motilin on I_CCh appears to be inversely correlated with the motilin concentration. As graphically summarized in Fig. 4B, the maximal effect of motilin was obtained at 1 nM, and the effect was dramatically diminished at higher concentrations. With 100 nM motilin, which contracts the cell and depolarizes the membrane maximally, in all cells examined the potentiation of I_CCh was no longer observed (0.86 ± 0.13, n = 4). On the other hand, when the concentration of motilin was fixed to produce the maximal potentiation effect (1 nM), the effect was most pronounced near the activation threshold for CCh (0.3–1 µM) but became marginal or even decremental at 100 µM CCh, which was sufficient to activate I_CCh maximally (Fig. 5A). To gain further insight into the mechanisms underlying this observation, we tested two extreme concentrations of CCh on the same cell. As illustrated in Fig. 5B, the amplitude of I_CCh induced by 1 µM CCh after motilin-induced potentiation was comparable to that of maximally activated I_CCh (with 100 µM CCh), thus suggesting that the potentiating effect of motilin may saturate when I_CCh is already fully activated.

Although more accurate quantification was not feasible due to cell-to-cell variation and time-dependent rundown of I_CCh, qualitatively the same results were ob-
tained from four other cells. We therefore speculate that saturation of motilin's effects on \( I_{\text{CCh}} \) reflects a leftward shift of the CCh-\( I_{\text{CCh}} \) activation curve (see Fig. 1). The speculated leftward shift of the CCh-\( I_{\text{CCh}} \) activation curve might result from increased sensitivity of the muscarinic receptors after motilin receptor stimulation. Consistent with this idea, the magnitude of the guanosine 5'-O-(3-thiotriphosphate)-induced cation current, which would reflect the opening of the same cation channels as activated by muscarinic receptor but bypassing the receptor (11, 15, 16, 30), was not significantly affected by motilin in its both developing and steady phases (Fig. 6A). The intracellular pathway(s) linking the motilin receptor may not involve the pertussis toxin-sensitive G protein, because no significant difference was found in the amplitude of \( I_{\text{Mest}} \) between cells treated with pertussis toxin (1 µg/ml, 36°C, 4–6 h) and those of time-matched control (data not shown).

These results suggest that the cellular mechanism underlying the potentiation of \( I_{\text{CCh}} \) by motilin might be different from that for the activation of \( I_{\text{CCh}} \), which is likely to involve the pertussis toxin-sensitive G protein (see above), but at present we have no definite evidence against the possibility that the pathway mediating the motilin-induced \( I_{\text{CCh}} \) potentiation may also be pertussis toxin sensitive.

The potentiating effect of motilin on \( I_{\text{CCh}} \) may involve some biochemical events such as enzymatic reactions. In accordance with this proposal, lowering the temperature to 20°C from 30–31°C almost completely abolished the enhancing effects of motilin (Fig. 6B).

**DISCUSSION**

The major finding of this study is that in rabbit duodenal smooth muscle cells potent amplification of the postsynaptic muscarinic responses (i.e., activation of inward currents) could be induced after the stimulation of motilin receptors, under conditions that exclude any contributions of nonmuscular factors. This amplification is specific for the muscarinic cation conductance...
because its characteristic features such as voltage dependence and sensitivity to atropine remained essentially unchanged after motilin-induced potentiation and could clearly be distinguished from those of the cationic conductance induced by motilin per se. This is of particular physiological significance because the muscarinic cation channels have been found ubiquitously in the whole gastrointestinal tract and are thought to play a central role in the excitatory regulation of the gut motility. Owing to the remarkable and dynamic dependence of the depolarizing actions of these channels on the membrane potential, intracellular free Ca$^{2+}$ concentration and probably the mechanical state (2, 5, 11–13, 15, 17, 18, 21, 28), they likely serve to tune finely the Ca$^{2+}$ spike activity (i.e., by altering its frequency and duration), a critical determinant for the kinetics of the gut motility (4, 6, 9, 17, 22).

The most interesting aspect of motilin-induced potentiation of $I_{\text{CCr}}$ is that the potentiation occurs maximally near the activation threshold of CCh (on average 3.5–5-fold with 0.3–1 µM CCh) and with a relatively low concentration of motilin (1 nM) that can itself induce only partial contraction (e.g., Fig. 1D in Ref. 29). In contrast, the potentiation diminished almost completely at higher concentrations of these agonists (Fig. 4 and 5). These results provide at least two important insights into the mechanism by which motilin exerts its complex actions under in vivo and in vitro conditions. First, the extent of motilin-induced amplification of postsynaptic cholinergic responses is likely to be closely associated with the prevalent in vivo parasympathetic tone. Thus, if we assume that parasympathetic tone is elevated after food intake and gradually declines during fasting, the inverse dependence of motilin-induced potentiation of $I_{\text{CCr}}$ on CCh concentration might serve most advantageously to enhance the gut motility when the parasympathetic tone has been decreased, i.e., during the fasting state. Conversely, this mechanism would become much less significant when the parasympathetic tone was elevated, e.g., during the progression of digestion. In this regard, it is interesting to note that the plasma motilin level is depressed during the postprandial period, whereas it starts to fluctuate in the fasting state, in an apparent association with the atropine-sensitive migrating motor complex (27). Such a temporally inversed relationship between the cholinergic nervous and plasma motilin activities might further help accentuate the postsynaptic amplification of muscarinic responses by motilin.

Second, the virtual resistance to atropine or tetrodotoxin pretreatment of motilin-induced contractions in vitro studies could partly be accounted for by our observation that high concentrations of motilin (100 nM) are unable to potentiate $I_{\text{CCr}}$ (Fig. 4B), whereas such concentrations can themselves, probably through inositol 1,4,5-trisphosphate-mediated Ca$^{2+}$ release and secondary activation of voltage-dependent Ca$^{2+}$ influx, provoke strong sustained contractions, the amplitudes of which are comparable to the maximal contractions induced by CCh (Fig. 1 in Ref. 29). In accordance with these observations, contractions induced by a near-threshold concentration of CCh (0.3 µM) tended to be enhanced after the application of 1 nM motilin (6–39% increase, n = 5), but this effect was dramatically reduced with higher concentrations of motilin (10–100 nM) or CCh (10–100 µM). Similar results have also been described by Strunz et al. (24) that the subthreshold concentration of 13-norleucine-motilin (a biologically active synthetic analog of motilin) reduces the acetylcholine dose required for producing half-maximal contractions by about 30-fold, with a slight increase in the maximal response (about 15%). Such a remarkable shift of dose-response relationship for CCh-induced contraction is indeed compatible with the leftward shift of dose-response curve for $I_{\text{CCr}}$ by motilin observed in the present study. This is probably accounted for by the increased sensitivity of the muscarinic receptor coupled to the cation channel (Fig. 6A). Although the in vivo relationship between the actions of motilin and the cholinergic system seems complicated by many factors, including their bidirectional interactions as well as the involvement of other intestinal peptides and/or unidentified nonneural factors (7, 8, 27), we would like to emphasize at least that the inconsistency with respect to...
to atropine sensitivity between in vivo and in vitro experiments may arise in part from insufficient attention to the dose-dependent interactions between motilin and the muscarinic receptors at the postsynaptic level. Further carefully designed experiments are warranted to provide a less equivocal understanding of this current controversial issue.

We are grateful to Dr. A. F. Brading, Univ. Dept. of Pharmacology, Oxford, United Kingdom, Chugai Pharmaceutical, and Miyuki Yashikawa for improving our manuscript, kindly providing us with GM109, and technically assisting us, respectively.

Portions of this study were presented at the Annual Conference of the Japanese Pharmacological Society in Makuhari, Chiba, Japan, in March, 1997.

Address reprint requests to R. Inoue.

Received 9 June 1997; accepted in final form 30 November 1997.

REFERENCES


