Effect of Trimebutine on Contractile Responses in Skinned Ileal Smooth Muscle

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ABSTRACT—The effects of trimebutine on Ca²⁺ release and modulation of Ca²⁺ sensitivity of contractile elements induced by carbachol (CCh) were investigated using a tension measuring method in β-escin-treated skinned smooth muscle of the longitudinal muscle layer of guinea pig ileum. Trimebutine (10⁻¹⁰ to 10⁻⁶ M) concentration-dependently inhibited tension development brought about by Ca²⁺ release from intracellular stores induced by CCh (10⁻⁷ M), but did not affect those induced by inositol 1,4,5-trisphosphate (IP₃, 25 μM) or caffeine (5 mM). The inhibitory effect was reversible. Trimebutine (100 μM) neither altered the Ca²⁺ sensitivity of the contractile elements nor affected the effects of GTPγS (50 μM) and CCh (100 μM) in potentiating Ca²⁺ sensitivity of the contractile elements after the Ca²⁺ storage function had been eliminated by A23187. These results suggest that trimebutine inhibits CCh-induced Ca²⁺ release by acting at some point during the coupling of muscarinic receptors through a G-protein to phospholipase C and thus reducing the accumulation of IP₃.

Keywords: Trimebutine, Skinned smooth muscle, Muscle contraction, Calcium storage, Calcium sensitivity

In vascular and intestinal smooth muscle, many excitatory agonists have been shown to induce not only Ca²⁺ mobilization from intracellular stores (Ca²⁺ release) but also potentiation of the contractile element Ca²⁺ sensitivity (Ca²⁺ sensitization). Their contractile effects, especially those without any change in the membrane potential (1), are considered to be attributable to both actions. It is now well established that the Ca²⁺ release arises from stimulation of a G-protein-coupled phosphatidylinositol cascade leading to hydrolysis of phosphatidylinositol 1,4-bisphosphate (PIP₂), of which a metabolite, inositol 1,4,5-trisphosphate (IP₃), releases Ca²⁺ from the intracellular stores (2). The signal transduction pathway through which the Ca²⁺ sensitization is brought about has not been clarified yet. One idea is that diacylglycerol, the counter metabolite of PIP₂, is involved in the Ca²⁺ sensitization (3, 4).

Kobayashi et al. (5) recently found that Ca²⁺-channel blockers, nifedipine and verapamil, can distinguish between the Ca²⁺ release and the Ca²⁺ sensitization mediated by α-adrenoceptors in portal vein smooth muscle; the Ca²⁺-channel blockers inhibit phenylephrine- and GTPγS-induced Ca²⁺ release without affecting phenylephrine- and GTPγS-induced Ca²⁺ sensitization. The results suggest that the adrenoceptor-mediated Ca²⁺ sensitization may be brought about through a signal transduction pathway independent of that for the Ca²⁺ release.

Trimebutine, which is available for treatment of both hypermotile and hypomotile gastrointestinal tract, including the irritable bowel syndrome and dyspepsia (6–8), stimulates or inhibits the mechanical activity depending on its level in the isolated stomach and intestine (9–11). Our previous studies demonstrated that trimebutine interacts with dihydropyridine binding sites in an allosteric manner (12) and blocks voltage activated Ca²⁺ current (13). Furthermore, trimebutine inhibits the muscarinic receptor-mediated cytoplasmic Ca²⁺ release from intracellular stores (14). Therefore, it is possible that trimebutine can discriminate the signal transduction pathway for the Ca²⁺ sensitization from the well-known signal transduction pathway for the Ca²⁺ release, as does the Ca²⁺-channel blockers (5).

To test this possibility, the present study was undertaken using a tension measuring method in β-escin-treated skinned smooth muscle of the longitudinal muscle layer of guinea pig ileum. The skinned smooth muscles with
\(\beta\)-escin have been widely used for studies on \(Ca^{2+}\) release and \(Ca^{2+}\) sensitization produced by various agents (2, 15, 16).

MATERIALS AND METHODS

Male guinea pigs (Hartley, 300–450 g) were stunned and bled to death. A 5-cm length of ileum was removed, and the longitudinal muscle layer of the intestinal segment was peeled off the underlying circular muscle. Under a binocular microscope, a muscle strip (5 mm in length, 0.3 mm in width) was dissected from the longitudinal muscle layer. Both ends (about 1 mm in length) of the muscle strip were firmly wrapped in small pieces of aluminium foil (1 \(\times\) 2 mm). A pin hole was bored in the empty region of the foils.

The muscle strip was mounted horizontally in a 0.3-ml organ bath with one end of it fixed on the rubber bottom of the bath with a pin, and the other attached to the thin lever of an isometric force transducer (TB-612; Nihon Kohden, Tokyo). The organ bath was filled with a physiological salt solution (PSS, composition given below) kept at 23°C, and the muscle strip was equilibrated for 30–60 min under a tension of 150–180 mg. When \(\beta\)-escin was used to achieve skinning of the muscle cells (2), contractile responses to 10 \(\mu\)M carbachol (CCh) were observed, and then the muscle strip was incubated with \(\beta\)-escin (40 \(\mu\)M) in a relaxing solution containing 4 mM EGTA (G4-solution, composition given below) for 30 min. After skinning, the bath medium was replaced with fresh G4-solution. If necessary, the skinned ileal muscle was treated with A23187 (10 \(\mu\)M) for 10 min to eliminate the intracellular \(Ca^{2+}\) storage function (17).

Intracellular \(Ca^{2+}\) stores of the skinned ileal muscle were loaded with \(Ca^{2+}\) by replacing G4-solution with a \(Ca^{2+}\)-containing solution (pCa 6) for 10 min. CCh, caffeine and IP3 were applied by replacing the bath medium (G4-solution) with 0.05 mM EGTA-containing relaxing solution (G0.05-solution, composition given below) to which a drug was added. After the drug application for 1–2 min, the drug was removed by reintroduction of G4-solution. When a trial of \(Ca^{2+}\)-loading and subsequent application of drugs was repeated in one preparation, the interval between two successive trials was 10–15 min.

PSS was a HEPES-buffered, modified Krebs solution containing: 126 mM NaCl, 6 mM KCl, 2 mM CaCl2, 1.2 mM MgCl2, 14 mM glucose and 10.5 mM HEPES, (pH adjusted to 7.2 with NaOH at room temperature). The composition of relaxing solution was: 130 mM K propionate, 4 mM MgCl2, 4 mM Na2ATP, 20 mM tris-maleate, 10 mM creatine phosphate, creatinine phosphokinase (3.3 units/ml), and 4 mM (for G4-solution) or 0.05 mM EGTA (for G0.05-solution) (pH adjusted to 6.8 with KOH). Solutions containing various \(Ca^{2+}\) concentrations were prepared by adding appropriate amounts of \(CaCl_2\) to the relaxing solution containing 2 mM EGTA. The \(Ca^{2+}\) concentration in these solutions was estimated using the apparent binding constant of 10⁶ M⁻¹ for the \(Ca^{2+}\)-EGTA complex, which was estimated at pH 6.8 and with an ionic strength of 0.16 at 25°C (3).

Drugs used were guanosine triphoshate (GTP), guanosine 5',7-O-[3-thiotriphosphate] (GTP7S), \(\beta\)-escin, inositol 1,4,5-trisphosphate (IP3), all which were purchased from Sigma (St. Louis, MO, USA); caffeine (from Wako, Osaka); carbachol chloride (CCh; from Tokyo Kasei, Tokyo); and trimebutine maleate (Tanabe, Osaka). All other chemicals used were of reagent grade.

Data were expressed as the mean ± S.E.M. and statistical significance was determined by Student's t-test. Probabilities less than 5% were considered significant.

RESULTS

Effect of trimebutine on CCh-induced release of \(Ca^{2+}\) stores

During \(Ca^{2+}\)-loading with a \(Ca^{2+}\)-containing (pCa 6) solution for 10 min (see Methods), a sustained rise in tension occurred (see Figs. 1 and 2). CCh (10 \(\mu\)M) in G0.05-solution, applied 4 min after reintroduction of G4-solution, produced a rapid rise in tension (Fig. 1A). The CCh-induced tension reached a peak within 1 min and then started to decline gradually in the continuing presence of CCh. The CCh-induced tension development has been reported to be due to a massive release of \(Ca^{2+}\) stores upon stimulation of the muscarinic receptors (2). The effect of CCh showed a small time-dependent rundown, but was fairly reproducible. The average peak tensions of the second and third responses to CCh were 95.5 ± 2.2% (n = 4) and 90.7 ± 7.3% (n = 3) that of the first response, respectively. Trimebutine applied after the \(Ca^{2+}\)-loading, suppressed the CCh-induced tension development (Fig. 1A). The inhibitory effect of trimebutine was reversible, and the CCh-induced tension development was restored by washout of the drug (Fig. 1A). Figure 1B shows the average peak tensions of the second CCh responses in the absence and presence of 10 \(\mu\)M and 100 \(\mu\)M trimebutine, expressed as a percentage of the peak tension of the first CCh response. The difference between the values for the CCh responses in the absence (95.5 ± 2.2%, n = 5) and presence of 100 \(\mu\)M trimebutine (61.5 ± 1.8, n = 4) was statistically significant (P < 0.01).

These results suggest that trimebutine concentration-dependently inhibits the CCh-induced release of \(Ca^{2+}\) stores in the skinned smooth muscle.
Trimebutine on Skinned Smooth Muscle

Fig. 1. Effect of trimebutine on carbachol (CCh, 10 μM)-induced contraction in skinned smooth muscle of guinea pig ileum. After exposing the skinned muscle to a Ca²⁺-containing solution (pCa 6) for 10 min to load Ca²⁺ into the stores and then returning it to a Ca²⁺-free solution (G4, see Methods) for 4 min, CCh was applied in another Ca²⁺-free solution (G0.05) as indicated by the bar. The protocol for exposure to the different solutions is indicated on the line below (A). Ca²⁺ loading at pCa 6 and subsequent application of CCh were repeated three times (A) or twice (B). A, Tension responses to CCh before (left) and after addition of trimebutine (50 μM) as indicated by the bar (middle) and after its washout (right) are shown. The interval of two successive panels is 5 min. Note that trimebutine reversibly reduces the peak tension of the CCh-response. B, Summarized effects of trimebutine on the CCh response. The amplitude of the 2nd CCh responses in the absence (Cont., open column) and presence of trimebutine (10 μM, closed column; 100 pM, shaded column) was expressed by taking the amplitude of the respective 1st CCh responses as 1.0. Each column indicates the mean±S.E.M. (shown by vertical bars) of 4-5 experiments. **P<0.01 vs. Cont. (open column).

Effects of trimebutine on caffeine- and inositol 1,4,5-trisphosphate-induced release of Ca²⁺ stores

Two types of Ca²⁺ release mechanism have been reported to operate in Ca²⁺ mobilization from the intracellular stores of smooth muscle, namely, Ca²⁺-induced Ca²⁺ release (CICR) and inositol 1,4,5-trisphosphate induced Ca²⁺ release (IICR, ref. 18). The CICR mechanism is sensitive to caffeine.

To determine the action of trimebutine on these two Ca²⁺ release mechanisms, the effects of the drug on caffeine- or IP₃-induced contractile responses were examined by the same procedure as used for CCh (19, 20). Caffeine (5 mM) or IP₃ (25 μM) in G0.05-solution, applied four min after Ca²⁺-loading, evoked a transient rise in tension (Fig. 2, A and B). The responses to caffeine and IP₃ were also fairly reproducible. The relative peak tension of the second response to the first one was 99.3±3.5% (n=3) for caffeine and 94.3±5.3% (n=3) for IP₃. As shown in Fig. 2, trimebutine (100 μM) had no effect on the caffeine- and IP₃-induced tension developments. The peak tension of the second response in the presence of trimebutine was 88.3±3.4% (caffeine, n=3) and 103.7±4.5% (IP₃, n=3) that of the respective first responses.

These results suggest that trimebutine does not affect the two Ca²⁺ release mechanisms, CICR and IICR.

Effect of trimebutine on the Ca²⁺ sensitivity of contractile elements

The effects of trimebutine on the Ca²⁺ sensitivity of contractile elements in the absence and presence of GTP₇S or CCh were investigated after the Ca²⁺ storage function of the store sites had been eliminated by A23187.

Trimebutine (10 or 100 μM), when applied during a sus-
tained rise in tension induced by Ca\(^{2+}\)-containing solution (pCa 6), had no appreciable effect on it (Fig. 3A). When GTP\(_{1S}\) (50 nM), an activator of GTP binding protein (2, 3, 21), was applied during a sustained tension development induced by Ca\(^{2+}\) containing solution (pCa 6.3), an additional rise in tension was elicited that reached a new sustained level within 2 min, as shown in Fig. 3B. Trimebutine (100 nM) did not have any apparent effect on the augmentation of the tension induced by GTP\(_{1S}\) (Fig. 3B).

CCh (100 nM), when applied during a sustained rise in tension induced by pCa 6.3 solution, produced an increase in Ca\(^{2+}\)-induced tension (Fig. 4A). When the same procedure was repeated at a 20-min interval in one muscle preparation, the peak tension of the second Ca\(^{2+}\) response in the presence of CCh decreased to 82.3 ± 5.2% (n=3) that of the first response (Fig. 4, A and B). Trimebutine (100 nM) did not significantly alter the Ca\(^{2+}\) response in the presence of CCh (Fig. 4A); the peak

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**Fig. 2.** Effects of trimebutine (100 nM) on caffeine (5 mM)- and inositol 1,4,5-trisphosphate (IP\(_3\))-induced contraction in skinned smooth muscle. Ca\(^{2+}\) loading at pCa 6 and subsequent application of caffeine (A) or IP\(_3\) (B) as indicated by the bar were repeated twice at a 24-min interval in four different preparations. The second application of caffeine or IP\(_3\) was made in the absence (upper in A and B) and presence of trimebutine (lower in A and B) as indicated by the bar. Note that there was no appreciable effect of trimebutine on the caffeine and IP\(_3\) responses.
tension of the second Ca$^2+$ response after treatment with trimebutine was 83.0±2.5% (n=3) that of the first response (Fig. 4B).

These results suggest that trimebutine does not affect the Ca$^2+$ sensitivity of the contractile elements in the absence and presence of GTP$_7$S or CCh in the intestinal smooth muscle.
DISCUSSION

The inhibition by trimebutine of the CCh-induced tension development in a Ca\(^{2+}\)-free environment is due to attenuation of the muscarinic receptor-mediated Ca\(^{2+}\) release from intracellular stores (Ca\(^{2+}\) release), since the drug had no effect on the Ca\(^{2+}\) sensitivity of the contractile elements (Ca\(^{2+}\) sensitization). This is consistent with the previous finding that trimebutine reduces a transient increase of intracellular Ca\(^{2+}\) due to release of Ca\(^{2+}\) stores by CCh in intact intestinal smooth muscle (14). The following signal transduction takes place to release Ca\(^{2+}\) from its intracellular store sites: stimulation of muscarinic receptors, conversion of G-protein associated with phospholipase C (PLC) from a GDP-binding to GTP-binding state, activation of PLC, accumulation of IP\(_3\), resulting from hydrolysis of phosphatidylinositol 1,4-bisphosphate (PIP\(_2\)), binding of IP\(_3\) to its receptor in the membrane of Ca\(^{2+}\) stores and opening of IICR-related channels. The failure of trimebutine to alter the IP\(_3\)-induced tension development suggests that the drug has no effect on the IICR mechanism and activity of enzymes to inactivate IP\(_3\), such as IP\(_3\)-5-phosphohomonoesterase.

Trimebutine appears to produce no appreciable change in the capacity of the Ca\(^{2+}\) store to retain Ca\(^{2+}\), as judged by its failure to reduce the caffeine-induced tension development as well as the IP\(_3\)-induced tension development. A \(^{3}H\)-methylscopolamine binding study has revealed the lack of antagonistic activity of trimebutine on the muscarinic (M\(_\text{1}\)-subtype) receptor (unpublished data, M. Nagasaki). Thus, it is possible that trimebutine acts at some point during the coupling of muscarinic receptors through a G-protein to PLC and thus reduces the accumulation of IP\(_3\). Further studies are needed to determine the site of action of trimebutine. The dissociation by trimebutine of the muscarinic Ca\(^{2+}\) release from the muscarinic Ca\(^{2+}\) sensitization does not favor the idea that the Ca\(^{2+}\) sensitization is mediated by diacylglycerol formed from PIP\(_2\).

Recently, Ca\(^{2+}\)-channel blockers such as verapamil and nifedipine have been reported to inhibit the Ca\(^{2+}\) release from intracellular store sites, but do not affect the Ca\(^{2+}\) sensitivity of contractile elements induced by phenylephrine, an \(\alpha\)-adrenoceptor agonist, in permeabilized portal vein smooth muscle (5). Thus, trimebutine is similar to the Ca\(^{2+}\)-channel blockers in distinguishing between receptor agonist-mediated Ca\(^{2+}\) release and Ca\(^{2+}\) sensitization. In addition, trimebutine blocks voltage-gated Ca\(^{2+}\) channels through negative allosteric interactions with 1,4-dihydropyridine binding sites (12, 13). Binding of trimebutine to 1,4-dihydropyridine binding sites on voltage-gated Ca\(^{2+}\) channels and/or resultant blockade of the Ca\(^{2+}\) channels or an unidentified process might modify CCh-mediated phosphatidylinositol turnover, resulting in a decrease in IP\(_3\) accumulation, as suggested for the Ca\(^{2+}\)-channel blockers (5).

Procaine inhibits Ca\(^{2+}\) release from intracellular stores by caffeine in arterial smooth muscle (22). The inhibitory action of trimebutine on the Ca\(^{2+}\) stores might be brought about through activation of the same mechanism as that of procaine action, because of its similarity in chemical structure. However, the present finding that trimebutine had no effect on caffeine-induced Ca\(^{2+}\) release makes this unlikely.

In smooth muscle, both Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) entry through Ca\(^{2+}\) channels in the plasma membrane are thought to be important sources of Ca\(^{2+}\) for contraction (e.g. 23, 24). The duality of the action of trimebutine on Ca\(^{2+}\) mobilization to block voltage-gated Ca\(^{2+}\) channels and to reduce release of Ca\(^{2+}\) stores may partly explain the inhibitory effects of trimebutine on gastrointestinal motility (9–11).

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