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Inhibitory Effects of Verapamil and TMB-8 on Tonic Contraction Are Accompanied by Inhibition of Phospholipase C Activity in Intact Gastric Smooth Muscle Cells

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=ABSTRACT=

Gastric smooth muscle of guinea pigs was used to investigate whether the inhibitory effect of calcium antagonists on tonic contraction was accompanied by inhibition of phospholipase C activity. Tonic contraction and [^{3}H]inositol phosphate (IP) formation in response to acetylcholine were measured after pretreatment with verapamil, nifedipine, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) or EGTA. Verapamil (10 μM), TMB-8 (10 μM) or EGTA (2 mM) significantly inhibited acetylcholine (1 μM)-stimulated tonic contraction but nifedipine (100 nM) did not. Acetylcholine dose-dependently increased the formation of [^{3}H]IP. This effect was not observed in the presence of 2 mM EGTA. Both verapamil and TMB-8 significantly inhibited [^{3}H]IP formation induced by 10 μM acetylcholine, whereas nifedipine did not. In a subsequent study, we measured phospholipase C activity in gastric muscle cell homogenate and in permeabilized cells to determine whether calcium antagonists could inhibit the activity directly. The calcium antagonists did not change the phospholipase C activity of the cell homogenate or the permeabilized cells. But EGTA decreased phospholipase C activity by 50%. These results suggest that the inhibitory effects of verapamil and TMB-8 on acetylcholine-stimulated tonic contraction may be accompanied by inhibition of phospholipase C activity.

Key Words: Verapamil, Nifedipine, TMB-8, Phospholipase C, Gastric muscle contraction

INTRODUCTION

The release of Ca^{2+} into the cytoplasm is generally believed to be related to excitation-contraction coupling in smooth muscle. The stored Ca^{2+} in the sarcoplasmic reticulum is released by inositol 1,4,5-trisphosphate (IP_3), an immediate product of receptor-operated phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase C. Several investigators have reported that agonist-stimulated contraction is accompanied by stimulation of inositol phospholipid metabolism (Baron et al, 1984; Somlyo et al, 1985; Chilvers et al, 1989; Gu et al, 1991). Furthermore, Hillemeier et

al (1991) showed that direct application of IP_3 caused contraction in permeabilized muscle cells isolated from feline stomach. There is, therefore, a close correlation between agonist-stimulated IP_3 formation and contraction in smooth muscle.

Calcium antagonists, such as verapamil and nifedipine, have been widely reported to relax vascular smooth muscle (Singh and Roche, 1977; Robinson et al, 1980; Theroux et al, 1980; Chew et al, 1981; Ono and Hashimoto, 1983) and to inhibit gastrin-releasing peptide-stimulated contraction of gastric smooth muscle cells of guinea pigs (Chijiiwa et al, 1991). Such inhibitory action of calcium antagonists was mainly considered to be due to blockage of extracellular calcium influx. Calcium dependence of phospholipase C activity, however, has been reported in permeabilized erythrocytes (Morris et al, 1990), and confirmed in a study using purified phospholipase C from bovine brain (Ryu et al, 1987). Phospholipase C activity can also be activated by sodium channel activators (Gusovsky et al, 1987) or by membrane depolarization induced by K^+ (Kendall and Nahorski, 1985) in the cerebral cortex. Such an activation might be explained by an increase in intracellular Ca^{2+} concentration via a voltage-dependent calcium channel. Given that phospholipase C activity is apparently calcium dependent, it could be assumed that the inhibitory effects of calcium antagonists on tonic contraction may be accompanied by inhibition of phospholipase C activity. In the present study, therefore, we investigated the effects of calcium antagonists on tonic contraction and phospholipase C activity in gastric smooth muscle cells of guinea pigs.

METHODS

Materials

Verapamil, nifedipine, acetylcholine bromide, 8-(N,N-diethylamino) octyl 3,4,5-trimethoxybenzoate (TMB-8), ethyleneglycol bis-(ß-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA), soybean phosphatidylinositol, streptolysin O, collagenase, soybean trypsin inhibitor, LiCl and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) were purchased from Sigma Chemical, St. Louis, MO; [^{3}H]inositol and [^{3}H]phosphatidylinositol from New England Nuclear, Boston, MA; Dowex AG 1X2 from Bio-Rad, Richmond, CA. Coomassie blue solution was obtained from Pierce Chemical, Lockford, IL.

Measurement of contractile response

Guinea pigs were killed by cervical dislocation and the stomachs quickly removed. The stomach was rinsed with ice-cold Krebs bicarbonate solution (mM: 120.8 NaCl, 4.5 KCl, 15.5 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 11.2 dextrose). The Krebs bicarbonate solution was aerated with 95% O_2-5% CO_2 until pH equalled 7.4. Connective tissue and mucous membrane were removed. Small muscle strips (2 × 10 mm) from the body portion were used to measure the contraction in a cylinder-shaped muscle chamber (40 ml capacity) filled with Krebs bicarbonate solution. The solution of the chamber was kept at 37°C and bubbled with a mixture of 95% O_2 - 5% CO_2 at pH 7.4. To record the isometric contraction, the lower end of the muscle preparation was anchored to a steel hook and the upper end to a force transducer (FT.03, Grass Instruments Co., Quincy, MA) connected to a Grass 7E polygraph. The preparation was loaded with a tension of 0.5 g, where it was kept for 30 min to equilibrate with the solution. The final concentration of acetylcholine was achieved by adding 0.04 ml acetylcholine to the chamber. Antagonists were administered 4 min prior to the treatment with acetylcholine.

Cell preparation

The isolated stomach was chopped into small segments (2 × 3 mm) and then washed twice with 30 ml of Krebs bicarbonate solution. Smooth muscle cells were isolated from guinea pig stomach according to Sim et al (1993). Briefly, the muscle segments were incubated for two successive 20 min periods at 37°C in 10 ml of Krebs bicarbonate solution containing 0.1% collagenase and 0.01% soybean trypsin inhibitor bubbled with 95% O_2-5% CO_2. The digestion solution was discarded and the residual segments of gastric smooth muscle were subjected to mild shearing force by slowly passing them through a wide-bore pipet. The cells were harvested by filtration through 500 μm Nitex and then washed three times with 20 ml of collagenase-free Krebs bicarbonate solution containing 0.1% bovine serum albumin (BSA). The cells were resuspended in 20 ml of Krebs bicarbonate solution containing 0.1% BSA and incubated with [^{3}H]inositol (10 μCi/ml, 71.6 Ci/mmol) for 150 min at 37°C. LiCl at a final concentration of 10 mM was then added to the solution, and the incubation continued for another 30 min. At the end of the incubation, the cells were washed three times with 10 ml of Krebs bicarbonate solution containing 20 mM Hepes, 10 mM LiCl and 0.1% BSA and were finally resuspended in 3 ml of the above solution.

Measurement of [^{3}H]inositol phosphates

Aliquots (90 μl; 560±125 μg of protein) of the resuspended muscle cells were incubated at 37°C for 20 min in the presence or absence of acetylcholine in a 100 μl volume. Calcium antagonists were added in 10 μl aliquots 4 min prior to the treatment with acetylcholine. The reaction was terminated by adding 200 μl of ice-cold 10% perchloric acid and then incubating on ice for 30 min. After centrifugation, 200 μl of the supernatant was neutralized by adding 140 μl of 1 N NaOH and diluted with 0.8 ml of distilled water. Total labeled inositol phosphates (IP) were assayed as described by Kim et al (1991). The diluted supernatants were applied to a Dowex AG 1×2 ion exchange column (100-200 mesh, formate form). The column was washed with 6 ml of distilled water (3×2 ml) and 6 ml of 60 mM ammonium formate-0.1 N formic acid to separate total IP from free inositol. Total IP were eluted with 2 ml of 1.5 M ammonium formate in 0.1 N formic acid and radioactivity was determined by liquid scintillation counting. The pellets were dissolved in 0.1 ml of 1 N NaOH and protein concentration was determined using Coomassie blue. [^{3}H] radioactivity due to IP was calculated per milligram of protein to correct for differences between preparations.

Preparation of permeabilized cells and cell homogenate

The dispersed gastric smooth muscle cells were permeabilized with 0.5 unit/ml of streptolysin O for 10 min at 37°C in a permeabilization medium (mM: 120 NaCl, 4.2 KCl, 1.2 KH_2PO_4, 0.8 $MgSO_4$, 20 Hepes, pH 7.4, 1 EGTA, 10 glucose, 5 ATP and 0.05% BSA). Cells were then washed and resuspended in 2 ml of intracellular medium (mM: 120 KCl, 2 $MgCl_2$, 5 KH_2PO_4, 20 Hepes, pH 7.2, 1 EGTA, 2 ATP, 10 creatine phosphate, 20 units/ml creatine phosphokinase and $CaCl_2$ to give a free Ca^{2+} concentration of 1 μM) as previously described by Balla et al (1991). In other experiments, the dispersed gastric smooth muscle cells were homogenized by sonication at 50 W for 4 seconds in a lysis buffer containing 10 mM Tris-HCl, pH 7.2, 1 mM EGTA, 0.5 mM EDTA, 3 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM DTT and 2 μg/ml leupeptin on

ice. After centrifugation, protein concentration in the supernatant was adjusted to 3 mg/ml.

Assay of phospholipase C

For the measurement of phospholipase C activity of cell homogenate and permeabilized cells, assays were performed in 200 μl of reaction mixture containing 20,000 cpm of [^{3}H]phosphatidylinositol, 300 μM soybean phosphatidylinositol, 0.1% sodium deoxycholate, 3 mM $CaCl_2$, 1 mM EGTA, 50 mM Hepes, pH 7.0, with cell homogenate and permeabilized cells as a source of enzyme. Suspensions were incubated at 37°C for 5 min and terminated as described by Hofmann and Majerus (1982).

Statistical analysis

Rresults were represented as mean ± SD and were analyzed using analysis of variance (ANOVA). Differences between groups were determined with the Newman-Keuls test. The level of significance was set at 5%.

RESULTS

Effects of calcium antagonists on tonic contraction

Fig. 1 shows the isometric contraction induced by 1 μM acetylcholine after pretreatment with nifedipine, verapamil, TMB-8 or EGTA. The decrease of basal tone during pretreatment before adding acetylcholine was observed. Acetylcholine alone caused a tonic contraction by 0.32±0.04 g in gastric smooth muscle strips of guinea pigs. Verapamil, TMB-8 and EGTA significantly inhibited acetylcholine-stimulated tonic contraction by 71.3%, 78.2%, and 89.5%, respectively, while nifedipine had no effect (Fig. 2).

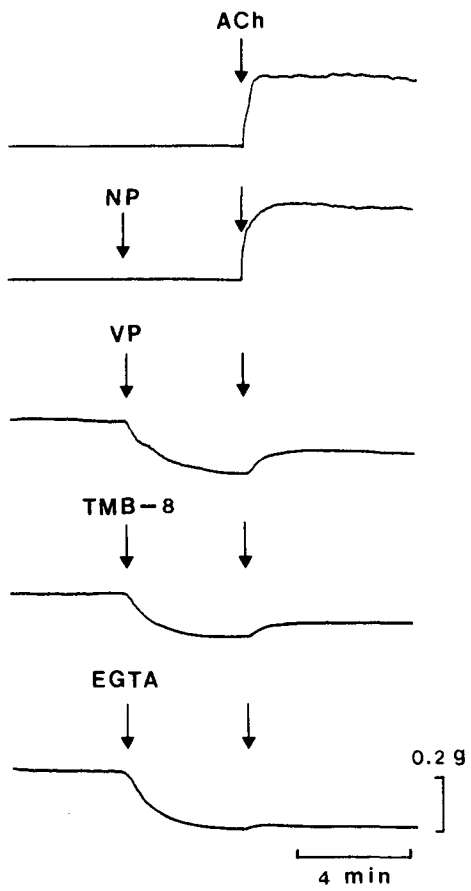

Fig. 1. Isometric contraction of gastric smooth muscle strips by acetylcholine (ACh, 1 μM) after pretreatment with nifedipine (NP, 100 nM), verapamil (VP, 10 μM), TMB-8 (10 μM) or EGTA (2 mM).

Formation of [^{3}H]IP in response to acetylcholine

To measure the formation of [^{3}H]IP induced by acetylcholine, 90 μl of [^{3}H]inositol-labelled cells was used. The suspension contained 640 ± 120 cpm/mg protein as measured by [^{3}H]IP radioactivity. Accumulation of [^{3}H]IP induced by acetylcholine is shown in Fig. 3. Acetylcholine increased the formation of [^{3}H]IP in a dose-dependent manner. However, in the presence of 2 mM EGTA, even high concentrations of acetyl-

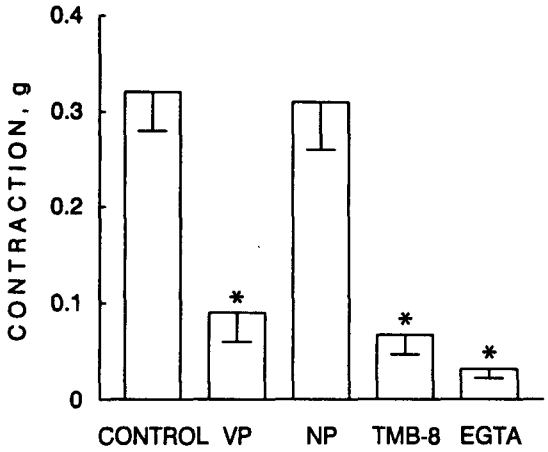

Fig. 2. The response of tonic contraction to acetylcholine (1 μM) after pretreatment with verapamil (VP, 10 μM), nifedipine (NP, 100 nM), TMB-8 (10 μM) or EGTA (2 mM). Control was tonic contraction induced by acetylcholine alone. Vertical bars indicate SD (n=8).
** P<0.05*

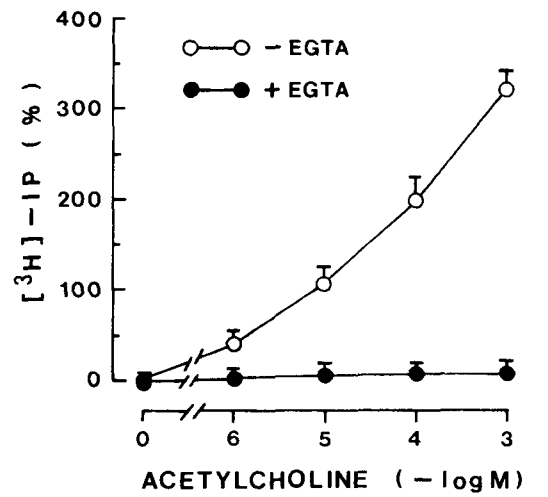

Fig. 3. Dose-response of [^{3}H]inositol phosphates (IP) to acetylcholine in the absence or presence of 2 mM EGTA in dispersed gastric smooth muscle cells of guinea pigs. Results indicate mean ± SD for six separate experiments and are expressed as percentage increases compared with unstimulated values (640±120 cpm/mg protein).

choline did not cause the formation of [^{3}H]IP, suggesting that acetylcholine-stimulated IP formation may be sensitive to extracellular Ca^{2+} in gastric smooth muscle cells of guinea pigs.

Effects of calcium antagonists on [^{3}H]IP formation

To examine the effects of calcium channel blockers on acetylcholine-stimulated [^{3}H]IP formation, [^{3}H]inositol-labelled gastric muscle cells were preincubated with verapamil, nifedipine or TMB-8 and then stimulated with 10 μM acetylcholine. As shown in Fig. 4, verapamil dose-dependently inhibited [^{3}H]IP formation induced by acetylcholine. However, nifedipine even at a concentration of 1 μM did not affect [^{3}H]IP formation induced by acetylcholine. Another calcium channel blocker, TMB-8, which is known to inhibit calcium release from intracellular stores, dose-dependently inhibited the formation of [^{3}H]IP induced by acetylcholine. However, none of these calcium antagonists alone had any effect on [^{3}H]IP formation under unstimulated, basal conditions. To exclude the possibility that the lack of effect of nifedipine may be due to the inactivation by light, we measured the phasic contraction that is mainly dependent on extracellular calcium (Biancani et al, 1987; Hillemeier et al, 1991) in corporal gastric smooth muscle of cats. Nifedipine significantly inhibited phasic contraction induced by acetylcholine at a concentration of 3 nM and completely inhibited at 100 nM. Therefore, the lack of effect of nifedipine on both tonic contraction and phosphatidylinositol turnover is not likely to be due to chemical inactivation in gastric smooth muscle of guinea pigs.

Phospholipase C activity

To examine whether the activity of phospholipase C was directly affected by verapamil, nifedipine, TMB-8 or EGTA, we used cell homogenate

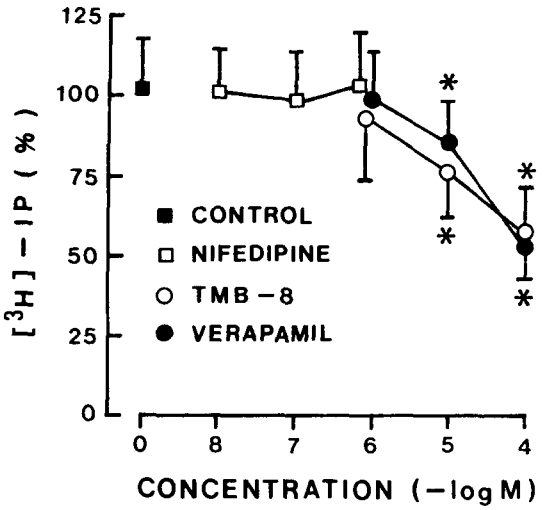

Fig. 4. Effect of verapamil, nifedipine or TMB-8 on acetylcholine-stimulated [^{3}H]inositol phosphate (IP) formation. The dispersed cells labelled with [^{3}H]inositol were preincubated with verapamil, nifedipine or TMB-8 for 4 min and then stimulated with acetylcholine (10 μM) for 20 min. The control value was percentage increase (102.3±16.6%) induced by acetylcholine (10 μM) alone. Vertical bars indicate SD (n=6).
** P<0.05*

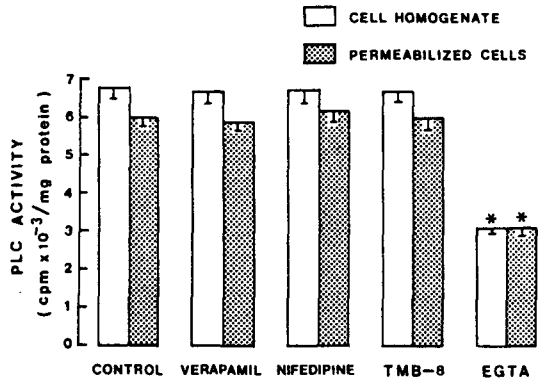

Fig. 5. Effects of calcium antagonists on phospholipase C (PLC) activity of cell homogenate and permeabilized cells. The cell homogenate (40 μl, 120 μg protein) and the permeabilized cells (40 μl, 160±10 μg protein) were incubated with phospholipase C substrate in the absence (control) or in the presence of verapamil (100 μM), nifedipine (1 μM), TMB-8 (100 μM) or EGTA (2 mM) for 4 min at 37℃. Vertical bars indicate SD (n=8).
** P<0.05*

(cytosolic phospholipase C) and permeabilized cells (membrane-associated phospholipase C). The activity of phospholipase C in both cell homogenate and permeabilized cells was assayed in the presence of verapamil (100 μM), nifedipine (1 μM), TMB-8 (100 μM) or EGTA (2 mM). The activity of phospholipase C in both cell homogenate and permeabilized cells was 6,830±360 and 5,940±210 cpm/mg protein, respectively. The activity of phospholipase C in cell homogenate and permeabilized cells was not affected by verapamil, nifedipine or TMB-8 (Fig. 5), suggesting that these calcium channel blockers did not act directly on phospholipase C. However, phospholipase C activity in both cell homogenate and permeabilized cells was significantly inhibited by 50% in the presence of 2 mM EGTA.

DISCUSSION

Our results suggest that verapamil and TMB-8 inhibit both tonic contraction and phosphatidylinositol breakdown induced by acetylcholine in gastric smooth muscle cells of guinea pigs. Acetylcholine-stimulated tonic contraction was significantly inhibited by verapamil or TMB-8 but not by nifedipine. According to previous reports that tonic contraction is mainly dependent on the release of calcium from intracellular stores by IP_3 (Biancani et al, 1987; Hillemeier et al, 1991), the inhibitory effect of TMB-8 on calcium release from intracellular stores (Hruska et al, 1986; Chijiiwa et al, 1991) can be explained. Among voltage-dependent calcium channel blockers that inhibit the influx of extracellular calcium, verapamil significantly inhibited acetylcholine-stimulated tonic contraction. Acetylcholine caused a small tonic contraction (about 10% of control) in the absence of extracellular calcium (2 mM EGTA). These results suggest that extracellular calcium may be involved in tonic contraction induced by acetylcholine. But

nifedipine did not inhibit acetylcholine-stimulated tonic contraction. The different effects between verapamil and nifedipine have been reported elsewhere (Mitra and Morad, 1985; Crews et al, 1988). Hagiwara et al (1993) also have suggested that verapamil inhibited Ca^{2+} influx that was not inhibited by felodipine or nifedipine. This difference may be responsible for different modes of action of phenylalkylamine and dihydropyridine (Catterall and Striessnig, 1992).

To examine whether the inhibitory effect of calcium antagonists on acetylcholine-stimulated tonic contraction may be accompanied by inhibition of phospholipase C activity, dispersed muscle cells labeled with [^{3}H]inositol were used to measure the formation of [^{3}H]IP induced by acetylcholine in the presence of verapamil, nifedipine, TMB-8 or EGTA. Acetylcholine increased [^{3}H]IP formation in gastric smooth muscle cells. The acetylcholine-stimulated IP formation did not occur in the absence of extracellular calcium, suggesting that phosphatidylinositol breakdown may be sensitive to extracellular calcium. This result contradicts the fact that receptor-activated phosphatidylinositol turnover is generally considered to be independent of extracellular calcium (Berridge, 1987). However, other studies have reported that phosphatidylinositol turnover is sensitive to extracellular calcium (Gonzales and Crews, 1984; Kendall and Nahorski, 1984). Verapamil and TMB-8 dose-dependently inhibited acetylcholine-stimulated [^{3}H]IP formation, and these effects on IP formation were similar to those seen on tonic contraction. These findings suggest that the inhibitory effects of verapamil and TMB-8 on tonic contraction may be due to the inhibition of phospholipase C activity. However, nifedipine did not inhibit IP formation and tonic contraction induced by acetylcholine.

To determine whether phospholipase C activity could be directly affected by calcium antagonists, we used both cell homogenate and permeabilized cells to measure phospholipase C activity, because multiple forms of phospholipase C have been purified from both particulate and soluble fractions of a variety of mammalian tissues (Rhee et al, 1989). In the present study, the activity of phospholipase C in cell homogenate and in permeabilized cells was not affected by verapamil, nifedipine or TMB-8. This observation suggests that the inhibitory action of these calcium channel blockers may be due to a decrease in cytosolic Ca^{2+} concentration rather than direct inhibition of phospholipase C itself.

We conclude that inhibitory effects of verapamil and TMB-8 on acetylcholine-stimulated tonic contraction may be accompanied by inhibition of phospholipase C activity in gastric smooth muscle cells of guinea pigs.

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