Possible Mechanisms of Action of the Antispasmodic Agent Tiropramide in the Isolated Detrusor from Rats

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ABSTRACT — The effects of tiropramide hydrochloride on Ca^{2+}-induced contraction, cytoplasmic free Ca^{2+} levels and tissue cyclic AMP concentrations were investigated to elucidate the mechanisms of its antispasmodic action in the isolated detrusor from rats. Tiropramide inhibited the Ca^{2+} (3 mM)-induced contractions of the isolated urinary bladder depolarized in a Ca^{2+}-free medium, and the IC_{50} value was 3.3×10^{-6} M. When tiropramide was added during the sustained phase of the K^+ (60 mM)-contracture, IC_{50} values of tiropramide for the contraction and the increased fluorescence were 1.9×10^{-5} M and 16.4×10^{-5} M, respectively. On the other hand, the IC_{50} values for the K^+-induced contraction and fluorescence after pretreatment of the isolated urinary bladder with tiropramide were 2.1×10^{-5} M and 2.6×10^{-5} M, respectively. Tissue cyclic AMP levels at 1 min after addition of 10^{-5} M tiropramide were significantly increased. Papaverine, IBMX or forskolin potentiated the inhibitory effect of tiropramide on carbachol-induced contraction and its cyclic AMP-elevating effect. However, a good correlation between the degrees of potentiation of the inhibitory effect and the increase in cyclic AMP levels was not observed. The present results suggest that the smooth muscle relaxant activity of tiropramide in the isolated detrusor from rats may be intimately associated with predominant inhibition of Ca^{2+} influx and, to a lesser extent, an increase in intracellular cyclic AMP levels.

Keywords: Urinary bladder (rat), Contraction, Tiropramide, Cytoplasmic free Ca^{2+}, Cyclic AMP

Tiropramide hydrochloride, (±)α-benzoylamino-(2-diethylamino-ethoxy)-*N*,*N*-dipropylbenzenepropanamide hydrochloride is pharmacologically characterized as a broad spectrum antispasmodic (1–3). Several mechanisms have been proposed to explain its action including a rise of tissue cyclic AMP levels by inhibition of phosphodiesterase and a subsequent increase in uptake of Ca^{2+} into the sarcoplasmic reticulum (4) and the inhibition of Ca^{2+} influx into the smooth muscle cell (1). There is, however, little information on the precise mechanisms of tiropramide in the isolated urinary bladder of rats. Therefore, we investigated the effects of tiropramide on the isolated detrusor from rats, with particular emphasis on simultaneous recordings of K^+-induced contraction and change in intracellular fura-2 fluorescence, and intracellular cyclic AMP levels. The effects of tiropramide on Ca^{2+}-induced contraction and those of a combination of tiropramide and cyclic AMP-elevating agents on carbachol-induced contraction and tissue cyclic AMP levels were also studied.

MATERIALS AND METHODS

Measurement of muscle tension

Male Wistar rats (7–9 weeks old) were killed by stunning and bleeding. The bladder dome was excised immediately, carefully dissected free from visible connective tissue and cut longitudinally into two strips (2 mm wide and 8 mm long). The strip was vertically suspended under a resting load of 1 g in a 10-ml organ bath filled with Krebs-Henseleit solution continuously gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C, and connected for recording of isometric contraction to a displacement transducer (UL-10GR, Minebea Co., Ltd., Nagano). The physiological solution had the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11.0 mM glucose. Af-

ter an equilibration period of 60 min, experiments were performed.

For the experiments of the combined effects of tiropramide with papaverine or forskolin, concentration-response curves for carbachol were constructed in a cumulative manner in the absence and presence of tiropramide, papaverine, forskolin, tiropramide plus papaverine, or tiropramide plus forskolin. The antispasmodics were preincubated for 5 min. The concentrations used were 1.5×10^{-5} M for tiropramide, 3×10^{-6} M for papaverine, and 10^{-6} M for forskolin; These concentrations corresponded to the IC_{30} for tiropramide and the IC_{10}s for papaverine and forskolin. All responses are expressed as a percentage of the maximal increase in tension induced by carbachol in the absence of the antispasmodics.

For the study of Ca^{2+}-induced contraction, after an equilibration period of 60 min in Krebs-Henseleit solution, the isolated urinary bladder strip was washed twice every 5 min in $CaCl_2$-depleted Krebs-Henseleit solution. The solution was then changed to the Ca^{2+}-free, depolarizing solution, which was obtained by replacing 118 mM NaCl by an equimolar amount of KCl. Ten minutes after that procedure, concentration-response curves for $CaCl_2$ were constructed in a cumulative manner. Tiropramide and terodiline were preincubated 5 min before the addition of $CaCl_2$.

Measurement of changes in cytosolic Ca^{2+} by fura-2

Cytoplasmic Ca^{2+} levels were measured using the Ca^{2+}-sensitive fluorescent dye fura-2. For this experiment, the urinary bladder dome was excised and cut longitudinally into four segments (2 mm wide and 7 mm long). The mucous membrane of the segments was removed. The segments were treated with the fura-2/AM at 5×10^{-6} M for 3 hr at room temperature in a darkened room. The fura-2 loading solution contained 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM HEPES, 5.5 mM glucose, 0.02% cremophore EL (BASF Japan) and 10 μM *N,N,N',N'*-tetrakis (2-pyridilmethyl)ethylenediamine (TPEN, Dojin, Kumamoto) (pH 7.4) (5).

After the fura-2 loading, the strip was spread out and fixed to a holder to minimize contractile movement. One end of the muscle strip was connected to a strain gauge transducer. Fluorescence measurements were carried out in a CAF-100 instrument (Jasco Co., Tokyo) equipped with a specially designed tissue bath (7 ml). Fura-2 fluorescence was measured at 340 and 380 nm (excitation) and 500 nm (emission), and the time course of the fluorescence change in the 380/340 nm ratio and the isometric muscle contraction (load: 1 g) were recorded simultaneously. The tissue was superfused with a physiological salt solution, equilibrated with 95% O_2 and 5% CO_2 and warmed (37°C), by means of a peristaltic pump at a flow rate of 7 ml/min. The salt solution had the following composition: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, and 5.5 mM glucose (pH 7.4). After a 20- to 40-min superfusion period, the responses of the detrusor to drugs were determined. The superfusion was interrupted 5 min before the determination and drugs were added in the tissue bath, bubbled with a mixture of 95% O_2 and 5% CO_2. The smooth muscle relaxants, tiropramide, terodiline and diltiazem, were introduced 5 min before or 5 to 10 min after 60 mM K^+ addition (hypertonic). The relaxant concentrations (IC_{50}) that halved the contractile response to 60 mM K^+ were calculated.

Determination of cyclic AMP content

Male Wistar rats (15 weeks old) were used. The bladder dome was cut longitudinally into four parts of 2×8 mm. The segments were equilibrated for 1 hr at 37°C in oxygenated Krebs-Henseleit solution. After incubation with the normal or drug-containing physiological salt solutions for 1, 3, or 10 min, the muscles were plunged into liquid nitrogen. The frozen tissues were then homogenized in cold 6% trichloroacetic acid (2 ml) at 4°C, and centrifuged at $2,000 \times g$ for 10 min. Supernatants were recovered and washed 5 times with 5 volumes of water saturated diethylether. The aqueous extracts remaining were lyophilized, the dried extracts were dissolved in assay buffer, and cyclic AMP was then assayed using Amersham's cAMP[^{125}I]assay system (dual range). Protein content was determined by the Lowry method (6) with bovine serum albumin as a standard.

Statistical analyses

All numerical data are expressed as the mean ± S.E. Tests of significance were performed by Student's *t*-test. A P value of less than 0.05 was considered to indicate a significant difference.

Drugs and chemicals

Tiropramide hydrochloride and terodiline hydrochloride were supplied by SmithKline Beecham Pharmaceuticals, Tokyo. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), diltiazem hydrochloride and papaverine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, and carbamylcholine chloride (carbachol) was from Tokyo Kasei, Tokyo. All drugs were dissolved in deionized water or a physiological salt solution (fluorescence measurement) except for forskolin. Forskolin was dissolved in ethanol and its final concen-

tration in the bathing solution never exceeded 0.4% (v/v). This concentration of ethanol little affected the carbachol-induced contraction of the isolated urinary bladder from rats and the control experiments were performed in the presence of an equal concentration of ethanol.

RESULTS

Combined effects of tiropramide with papaverine or forskolin

Figure 1 illustrates the inhibitory effect of tiropramide on the concentration-response curves for carbachol in the absence and presence of the cyclic AMP-elevating agents, papaverine and forskolin, in the isolated detrusor of rats. Tiropramide at a concentration of 1.5×10^{-5} M shifted the concentration-response curve downward, and papaverine (3×10^{-6} M) had little effect on the curve for lower concentrations of carbachol (Fig. 1A). A combination of tiropramide and papaverine potentiated the inhibitory effect of tiropramide on the contraction induced by carbachol (3×10^{-6} to 10^{-4} M). Forskolin at a concentration of 10^{-6} M displaced the concentration-response curve for carbachol to the right. Forskolin enhanced the relaxing effect of tiropramide at all concentrations of carbachol used.

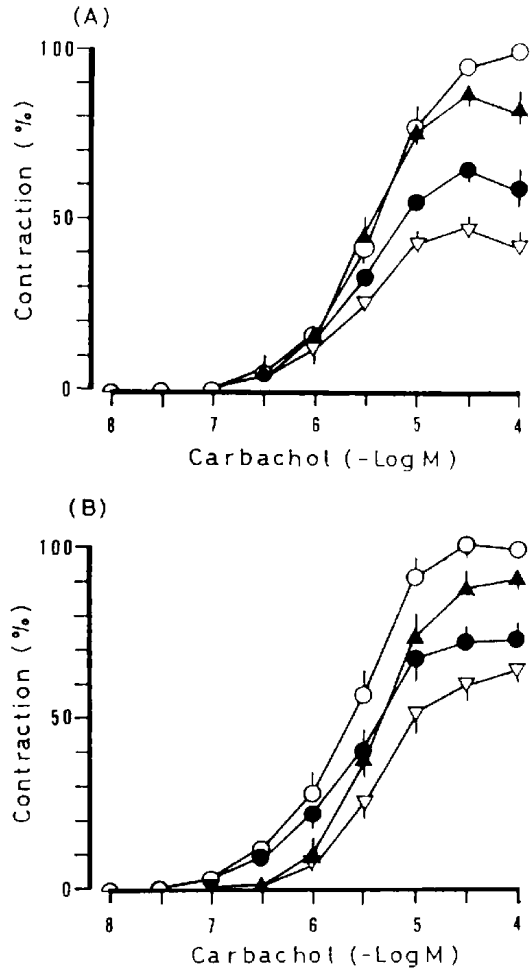

Fig. 1. Effects of tiropramide on concentration-response curves for carbachol in the absence and presence of cyclic AMP-elevating agents, papaverine (A) and forskolin (B), in isolated detrusor of rats. Vertical bars indicate ± S.E.M. of 6 experiments. In the absence of bars, twice the S.E.M. is less than the size of the printed symbol. ○: Control; ●: Tiropramide, 1.5×10^{-5} M; ▲: Papaverine, 3×10^{-6} M (A); Forskolin, 1×10^{-6} M (B); ▽: Tiropramide + Papaverine (A), Tiropramide + Forskolin (B).

Effects of tiropramide on Ca^{2+}-induced contraction

The effects of tiropramide and terodiline on cumulative concentration-response curves for $CaCl_2$ in the K^+-depolarized urinary bladder of rats are shown in Fig. 2. Contractions of the isolated urinary bladder by $CaCl_2$ in the Ca^{2+}-free, isotonic high K^+ medium were dose-dependently inhibited by tiropramide (3×10^{-6} M and 6×10^{-6} M), and terodiline (3×10^{-6} M, 10^{-5} M) inhibited dose-dependently Ca^{2+}-induced contractions (Fig. 2). IC_{50} values ($\times 10^{-6}$ M) of tiropramide and terodiline for the Ca^{2+} (3 mM)-evoked contractions were 3.3 ± 0.2 (N = 5) and 5.8 ± 0.5 (N = 6), respectively, and there was a significant difference between the two values ($P < 0.05$).

Effect of tiropramide on changes in $[Ca^{2+}]_i$

The effects of tiropramide on high K^+ (60 mM)-induced fluorescence and contractions measured simultaneously in the same detrusor from the rat are depicted in Fig. 3. Addition of hypertonic K^+ (60 mM) caused rises of the fluorescence and force development. After reaching their peak values, they declined and reached steady state levels (Fig. 3). The fluorescence increment preceded the force development. On washing out the K^+-rich medium, both fluorescence and force returned to their control values (data not shown). Tiropramide inhibited the increased fluorescence and contracture induced by the high K^+, independent of the pre- or post-treatment of the tissue with tiropramide (Fig. 3, A and B).

Table 1 summarizes IC_{50} values for tiropramide, terodiline and diltiazem when the smooth muscle relaxants were introduced 5 min before (pre-treatment) or 5 to 10 min after (post-treatment) the addition of 60 mM K^+. When tiropramide or terodiline was added during the sustained phase of the K^+-contracture, the IC_{50} values for tiropramide to the contraction and the increased fluorescence were 1.9×10^{-5} M and 16.4×10^{-5} M, respectively, and the respective IC_{50} values for terodi-

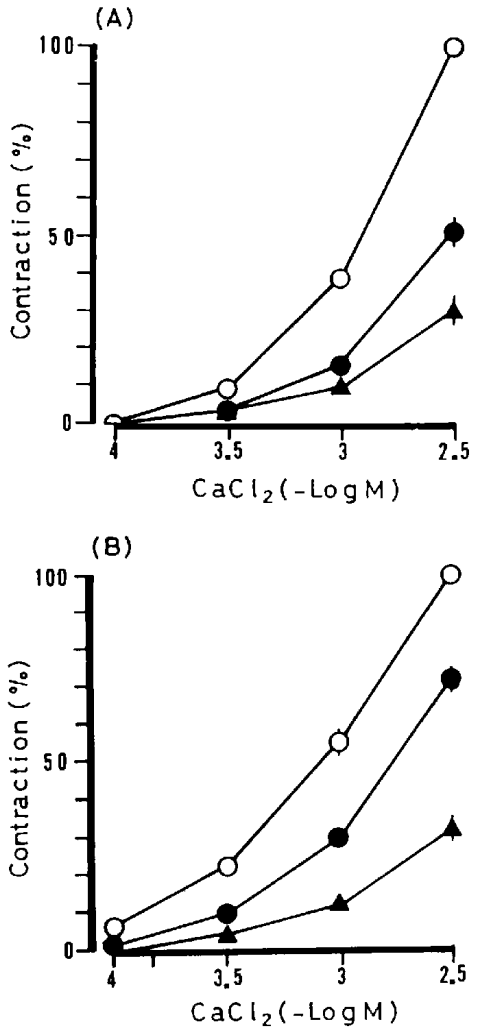

Fig. 2. Effects of tiropramide (A) (●: 3×10^{-6} M, ▲: 6×10^{-6} M) and terodiline (B) (●: 3×10^{-6} M, ▲: 1×10^{-5} M) on cumulative concentration-response curves for $CaCl_2$ (○: Control) in K^+-depolarized detrusor of rats. Vertical bars indicate ± S.E.M. of 5 to 6 experiments. In the absence of bars, twice the S.E.M. is less than the size of the printed symbol.

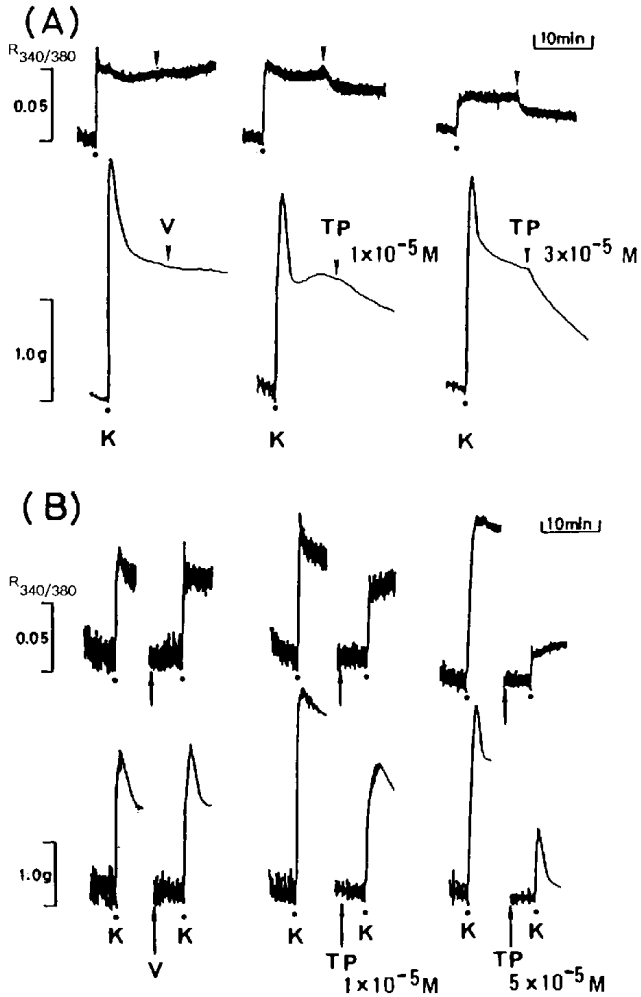

Fig. 3. Redrawing of original records, showing the effects of tiropramide on high K^+ (60 mM)-induced fluorescence and contractions measured simultaneously in the same detrusor of rats. Tiropramide was introduced 5 min before (A) or 5 to 10 min after (B) addition of 60 mM K^+. Upper traces represent changes in fluorescence and lower ones contraction. Changes in $[Ca^{2+}]_i$ are expressed by 340/380 nm ratios. V: vehicle, TP: tiropramide.

line were 0.6×10^{-5} M and 1.0×10^{-5} M (Table 1). The increased fluorescence was resistant to tiropramide to a greater extent as compared with the contraction ($P < 0.05$). On the other hand, the K^+-induced contraction and fluorescence after pretreatment of a urinary bladder strip with tiropramide were inhibited to the same extent, and no significant difference was observed between the IC_{50} values. The results obtained with terodiline and diltiazem were similar to those obtained with tiropramide, although diltiazem was more potent (Table 1).

Effect of tiropramide on tissue cyclic AMP levels

The effects of tiropramide (10^{-5} M), IBMX (10^{-5} M), forskolin (3×10^{-7} M), tiropramide plus IBMX, and tiropramide plus forskolin on intracellular cyclic AMP levels in the isolated detrusor from rats are presented in Fig. 4. Tiropramide at a concentration which influenced the tension of the isolated muscle induced increases of tissue cyclic AMP at all incubation time intervals (1, 3, and 10 min), but not time-dependently. The effect of IBMX on cyclic AMP levels was similar to that of tiropramide. Forskolin (3×10^{-7} M) increased cyclic AMP levels as function of time. The cyclic AMP-elevating effect of tiropramide was potentiated in the presence of IBMX (10^{-5} M) or forskolin (3×10^{-7} M).

Table 1. Effects of tiropramide, terodiline and diltiazem on K^+ (60 mM)-induced contraction and fluorescence in isolated urinary bladder of rats

	IC_{50} ($\times 10^{-5}$ M)[a]	
	Contraction	Fluorescence
Post-treatment[b]		
Tiropramide	1.9 ± 0.3 (4)	16.4 ± 5.8 (4)[d]
Terodiline	0.6 ± 0.1 (4)	1.0 ± 0.2 (4)
Pre-treatment[c]		
Tiropramide	2.1 ± 0.2 (4)	2.6 ± 0.8 (4)
Terodiline	1.6 ± 0.1 (5)	2.0 ± 0.7 (5)
Diltiazem	0.1 ± 0.02 (4)	0.3 ± 0.1 (5)

[a]: IC_{50} values are the concentrations required to reduce K^+-induced contraction or fluorescence by 50%. Values are given as the mean ± S.E. The figures in the parentheses are the number of rats. [b]: The smooth muscle relaxants were introduced 5 to 10 min after addition of 60 mM K^+. [c]: The relaxants were introduced 5 min before the addition of 60 mM K^+. [d]: Significantly different from the value for contraction at $P < 0.05$.

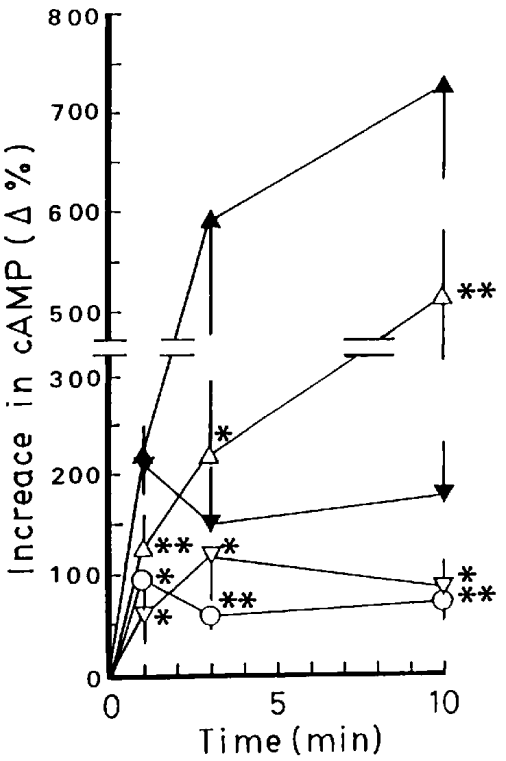

Fig. 4. Effects of tiropramide (○: 10^{-5} M), IBMX (▽: 10^{-5} M), forskolin (△: 3×10^{-7} M), tiropramide plus IBMX (▼), and tiropramide plus forskolin (▲) on intracellular cyclic AMP levels. Concentration of cyclic AMP at a basal level was 146.4 ± 11.2 pmol/mg protein (N = 5; mean ± S.E.M.). Vertical bars indicate ± S.E.M. of 4 to 5 experiments. *$P < 0.05$, **$P < 0.01$, compared with the basal value.

DISCUSSION

In the present study, tiropramide and terodiline inhibited the contractile response to Ca^{2+} in K^+-depolarized rat urinary bladder, suggesting that the possibility that their relaxation effect on the smooth muscle may be related to an inhibition of Ca^{2+} entry into the cell. Similar results have been obtained by Takayanagi et al. (1) in K^+-depolarized guinea pig taenia caecum. However, it has been reported (2) that tiropramide shows very small calcium channel blocking activity in vascular smooth muscles. These results indicate the different effects of tiropramide on the Ca^{2+} channels in different smooth muscles. The calcium antagonistic effects of terodiline have been demonstrated in several in vitro models (7–9). Our results suggest that the inhibitory effect of tiropramide on Ca^{2+}-induced contractions is more potent than that of terodiline in the isolated detrusor from rats.

The observations described above illustrate the usefulness of the Ca^{2+} indicator fura-2 for investigating the mechanisms of action of tiropramide in the urinary bladder of rats. Increases in fluorescence and force induced by K^+-depolarization were reduced by tiropramide. A good correlation between reductions of fluorescence and contraction was obtained when the rat urinary bladder was pretreated with tiropramide. Similar results were obtained with diltiazem, a calcium antagonist. When tiropramide was added during the sustained phase of the K^+-contraction, the fluorescence was reduced to a less extent compared to the contraction. On the other hand, the inhibitory effects of terodiline on the fluorescence and contraction were similar, independent of whether the tissue was pretreated or post-treated with terodiline. These results suggest that tiropramide predominantly inhibit Ca^{2+} entry into the cell via voltage-dependent Ca^{2+} channels in the urinary bladder of rats.

Vidal y Plana et al. (4) have suggested that the smooth muscle relaxant activity of tiropramide in the isolated rabbit colon arises from the drug-induced increase in tissue cyclic AMP concentrations possibly because of an inhibition of cyclic AMP catabolism. If the antispasmodic effect of tiropramide is closely linked to cyclic AMP-generation, a combination of low concentrations of a phosphodiesterase inhibitor or an adenylate cyclase activator with tiropramide should result in a great potentiation of the relaxing response to tiropramide. We investigated the combined effects of tiropramide with papaverine, a phosphodiesterase inhibitor, or forskolin, an adenylate cyclase activator, on contractile responses in the rat isolated detrusor. The effect of the combination of tiropramide and papaverine on the

concentration-response curve for carbachol was complex, and the curve was shifted downward at higher concentrations of carbachol. In contrast, forskolin enhanced the relaxing effect of tiropramide at all concentrations of carbachol used. These results suggest that the effect of tiropramide is differently influenced by the phosphodiesterase inhibitor and the adenylate cyclase activator. The cyclic AMP-increasing effect of tiropramide was also potentiated by IBMX or forskolin. Thus, the present data on the combined effects of the phospodiesterase inhibitors or forskolin with tiropramide on mechanical responses or intracellular cyclic AMP levels were so confusing that it was not possible to predict the precise mechanisms of the cyclic AMP-elevating action of tiropramide. The complex effects of a combination of tiropramide and papaverine may be due to multiple possible mechanisms of action of papaverine, such as phosphodiesterase inhibition (10, 11), inhibition of Ca^{2+} influx (12, 13) and Ca^{2+} release (13). It has reported that the effects of a combination of tiropramide and theophylline on cyclic AMP concentrations are additive and that tiropramide produced a dose-dependent inhibition of phosphodiesterase activity in the rabbit colon only at concentrations higher than those causing muscle relaxation (4). In the present study, tiropramide at a concentration of 10^{-5} M, which causes muscle relaxation, produced increases in the cyclic AMP levels (IC_{50} of tiropramide for carbachol-induced contraction = 3.6×10^{-5} M) (14). Our results and those of others suggest that the antispasmodic effects of tiropramide are, in part, related to increased intracellular cyclic AMP levels by an inhibition of phosphodiesterase activity.

Forskolin elevated cyclic AMP levels to a great extent at a concentration lower than that producing muscle relaxation. It would appear that a direct correlation between the amount of cyclic AMP produced and magnitude of the inhibitory response may not always be observed when comparing the response to papaverine. A possible explanation for this might be that forskolin could activate most, if not all, of the adenylate cyclase. Vegesna and Diamond (15) have suggested some form of functional compartmentalization of cyclic AMP in smooth muscle.

In conclusion, our results suggest that the smooth muscle relaxant activity of tiropramide in the isolated detrusor from rats is intimately associated with the predominant inhibition of Ca^{2+} influx and, to a lesser extent, the increase in intracellular cyclic AMP levels.

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