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The effect of Ca^{2+} antagonists on spontaneous motility from sheep duodenum

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Abstract—Longitudinal smooth muscle of the sheep duodenum showed a rhythmic spontaneous activity with an average frequency of $5{\cdot}6 \pm 0{\cdot}55$ phasic movements min^{-1} and a mean value of the amplitude of phasic contractions of $0{\cdot}956 \pm 0{\cdot}1$ g. When the strips were incubated in Ca^{2+}-free medium, the spontaneous motility amplitude (SMA) was reduced to $37 \pm 8{\cdot}2\%$ of control values. In Ca^{2+}-free medium plus EDTA (1 or 2 mM), the SMA was strongly reduced to $21{\cdot}9 \pm 8{\cdot}3$ and $1{\cdot}8 \pm 1{\cdot}8\%$, respectively. Verapamil, nifedipine and diltiazem diminished the SMA. The EC50 value for verapamil was 10^{-9} M, whereas that for diltiazem was 2×10^{-9} M and for nifedipine was 3×10^{-14} M. Trifluoperazine and TMB-8 reduced the SMA with EC50 values of 7×10^{-6} and 3×10^{-5} M, respectively. The spontaneous activity in the sheep duodenum seemed to be mediated by influx extracellular Ca^{2+}, which enters through potential-dependent channels and intracellular Ca^{2+} release.

The role of Ca^{2+} as a regulator of smooth muscle tension has already been established (Bolton 1979). It is now evident that significant differences exist in the degree of participation of intra- and extracellular Ca^{2+} pools during smooth muscle contraction. This depends on factors such as the type of smooth muscle (Karaki & Weiss 1984; Maggi et al 1985; Barone et al 1986; Marin 1988), the nature of the activating stimuli (Casteels & Raeymaekers 1979; Milanov et al 1984; Matthijs et al 1988; Parekh & Brading 1991) and the species being studied (Snape 1982; Arruebo et al 1987a,b).

In most gastrointestinal smooth muscles, calcium enters the cells from the extracellular fluid through calcium channels, pools that are tightly bound to the smooth muscle cell membranes, or caveoli (Yu & Bose 1991). Calcium channels can be divided into two categories: potential-dependent channels and receptor-operated channels (Bolton 1979; Karaki & Weiss 1984, 1988; Yu & Bose 1991). Voltage-dependent channels respond to changes in membrane potential evoked by chemical or electrical stimuli. Receptor-operated channels are insensitive to the changes in membrane potential and are activated by neurotransmitters, hormones and drugs (Bolton 1979; Yu & Bose 1991).

Calcium antagonists represent a heterogeneous class of agents, which may be defined as drugs that alter the calcium cellular function by inhibiting its entry from extracellular space or its release from cellular stores by interfering with one of its intracellular actions. The calcium entry from extracellular medium is either inhibited or blocked by the group of agents that includes phenylalkylamines, dihydropyridines and benzothiazepines. However, the calcium intracellular actions are reduced or suppressed by the calmodulin antagonists or inhibitors of intracellular calcium release (Godfraind et al 1986; Yu & Bose 1991; Spedding & Paoletti 1992).

Calcium ions and different Ca^{2+} antagonists have been investigated in the spontaneous activity of the guinea-pig taenia coli (Casteels & Raeymaekers 1979), in the contraction of colonic smooth muscle (Snape 1982), in the pendular movements of the rabbit ileum (Beleslin et al 1985), and in the rabbit duodenum (Coruzzi & Poli 1987). Since no more information is available on the role of calcium in the spontaneous activity of the sheep duodenal muscle, the main aim of the present study was to evaluate the action of extracellular and intracellular Ca^{2+} in the spontaneous activity of the sheep duodenum. We examined the effect of various Ca^{2+}-channel blockers and several drugs that act on Ca^{2+} at the intracellular level.

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Materials and methods

Muscle preparation. Segments of duodenum (1–4 cm) from the pylorus were rapidly removed from sheep immediately after slaughter in the abattoir and transferred to the laboratory in ice-cold Krebs solution within approximately 20 min. The duodenum was washed, double muscle layers were freed from mesenteric attachment and cut into longitudinal smooth muscle strips, 15 mm long and 5 mm wide. The strips were suspended in a thermostatically controlled organ bath of 10 mL capacity containing Krebs solution at 37°C, continuously gassed with 95% O_2–5% CO_2. The pH of the Krebs solution was 7·4. Mechanical activities were isometrically recorded by a strain gauge transducer connected to a pen recorder (Beckman R 511, IL, USA), the initial load being 2 g.

Experimental procedure. Strips were allowed to equilibrate in a normal Krebs solution for 60 min before use. Cumulative concentration-response curves to the various antagonists were constructed. The antagonists were added directly to the bath and the concentration was increased as soon as there was a stable response (approx. 5 min). Each strip was used for only one concentration-response curve.

Solutions and drugs. The composition of the normal Krebs solution was (mM): NaCl 120, KCl 4·7, $CaCl_2$ 2·4, $MgSO_4$ 1·2, $NaHCO_3$ 24·5, KH_2PO_4 1·0, glucose 5·6. Some duodenal preparations were exposed to a Ca^{2+}-free Krebs solution ($CaCl_2$ was omitted and substituted by equimolar NaCl), and in some cases, Ca^{2+}-free Krebs solution plus EDTA (1 or 2 mM) was used to check the effect of Ca^{2+} withdrawal on the spontaneous activity.

Verapamil hydrochloride, trifluoperazine dihydrochloride, ethylene diamine tetraacetic acid (EDTA), diltiazem, nifedipine, TMB-8 (8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of analytical grade.

All drugs were dissolved in distilled water except for nifedipine, which was dissolved in 95% ethanol and kept in the dark to avoid light-induced degradation. All nifedipine experiments were carried out in the dark.

Stock solutions of drugs were stored at −20°C and fresh dilutions were made daily. All drugs were added directly into the organ chambers in volumes of 50 μL. The concentrations reported are presented as the calculated final concentration in the bath solution.

Evaluation of data. Calculations of the mean effective concentrations (EC50, the concentration of the drug required to reduce the spontaneous activity amplitude to 50%) and the 95% confidence limits were calculated using linear regression according to the method of least-squares. In all cases, the spontaneous activity frequency was taken as an average over 5 min before and 5 min after treatment with the Ca^{2+} antagonists.

Analysis of variance was used for statistical comparisons, and

P values were determined by the Scheffe F-test. Values smaller than 0·05 were considered as being significantly different.

Results

After 60 min of adaptation, the longitudinal smooth muscle strips of the sheep duodenum in-vitro usually showed a rhythmic spontaneous activity, or pendular movements, with an average frequency of $5·6 \pm 0·55$ phasic movements min^{-1} and a mean value of the amplitude of phasic contractions of $0·956 \pm 0·1$ g (n = 31).

Effect of extracellular Ca^{2+}. In the first series of experiments, the longitudinal strips of the sheep duodenum were exposed to Ca^{2+}-free Krebs solution or Ca^{2+}-free medium plus EDTA. After exposing the preparations for 20 min in Ca^{2+}-free solution, the amplitude of movement was reduced to $84·6 \pm 10·6\%$ with respect to control (n = 45) and the frequency of movements was not modified (n = 45). When the preparations were incubated in Ca^{2+}-free solution for 60 min, the amplitude of movement was reduced to $37·6 \pm 8·2\%$ and the frequency of movements was diminished to $50 \pm 8·7\%$ with respect to control (n = 11, $P < 0·001$). These effects were reversible after washing out and incubation with Krebs solution.

Incubation of the duodenum strips in Ca^{2+}-free solution with EDTA (1 or 2 mM) strongly reduced the amplitude of movement to $21·9 \pm 8·3\%$ (n = 10, $P < 0·05$) and to $1·8 \pm 1·8\%$, respectively (n = 7, $P < 0·01$). The frequency of movements was reduced to $48·3 \pm 15·3\%$ (n = 10, $P < 0·05$) in the first case, and $13·0 \pm 13·0\%$ (n = 7, $P < 0·001$) in the second. The inhibitory effect was reversible after washing.

Effect of potential-dependent Ca^{2+} channel antagonists. In the second series of experiments, the effect of verapamil (10^{-11}–10^{-5} M), diltiazem (10^{-10}–10^{-4} M) and nifedipine (10^{-14}–10^{-8} M) on the amplitude and frequency of spontaneous activity of the duodenum was investigated in Krebs solution. Verapamil, diltiazem and nifedipine reduced or abolished the amplitude of movements (Fig. 1). The inhibitory effect of verapamil, diltiazem and nifedipine on the motility amplitude was concentration-dependent. The EC50 for verapamil was 10^{-9} M, whereas that for diltiazem was 2×10^{-9} M, and for nifedipine was 3×10^{-14} M (Table 1). The potency of nifedipine was approximately of 30000-fold that of verapamil and 60000-fold that of diltiazem. After washing, the inhibition was reversible to nifedipine but not to diltiazem or verapamil. When the preparations had a small spontaneous activity, the addition of diltiazem caused relaxation responses (data not shown).

The frequency of movements was reduced, but not significantly, at the highest concentration of verapamil, but it was blocked at the highest concentration of diltiazem and nifedipine ($P < 0·001$).

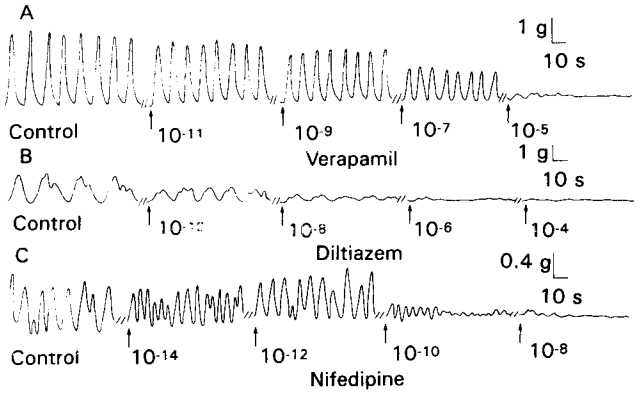

FIG. 1. Effect of A. verapamil, B. diltiazem and C. nifedipine on spontaneous activity in sheep duodenum. Arrowheads indicate addition of drugs.

Table 1. EC50 values of Ca^{2+} antagonists on motility amplitude in sheep duodenum.

Agents	EC50 (M)	n
Verapamil	$1·2 \pm 0·3 \times 10^{-9}$*	14–16
Diltiazem	$2·0 \pm 0·0 \times 10^{-9}$*	11–14
Nifedipine	$3·5 \pm 0·0 \times 10^{-14}$	11–12
Trifluoperazine	$7·7 \pm 0·4 \times 10^{-6}$*	13–15
TMB-8	$3·4 \pm 0·0 \times 10^{-5}$*	13–15

The values are expressed in the mean ± s.e. * $P < 0·05$.

Effect of intracellular Ca^{2+} inhibitors. In the third series of experiments, the effect of trifluoperazine (10^{-7}–10^{-4} M) and of TMB-8 (10^{-8}–10^{-4} M) on the spontaneous motility of the duodenum was tested in Krebs medium (Fig. 2). Trifluoperazine and TMB-8 inhibited the motility amplitude and were concentration-dependent. The EC50 for trifluoperazine was 7×10^{-6} M, whereas that for TMB-8 was 3×10^{-5} M (Table 1). The potency

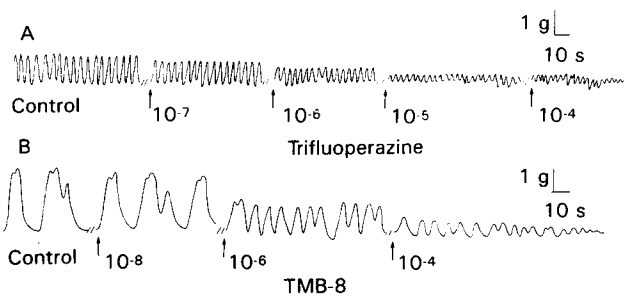

FIG. 2. Effect of A. trifluoperazine and B. TMB-8 on spontaneous activity in sheep duodenum. Arrowheads indicate addition of drugs.

of trifluoperazine was 4-fold more potent than that of TMB-8 in reducing the motility amplitude. The inhibition was reversible after washing for both compounds. On the other hand, neither trifluoperazine nor TMB-8 modified the frequency of spontaneous activity.

The present results show that the inhibition of spontaneous motility amplitude is higher with verapamil, diltiazem and nifedipine (Ca^{2+}-channel blockers) than with drugs that interfere with intracellular Ca^{2+} (trifluoperazine and TMB-8).

Discussion

Results obtained in the present study show the role of extracellular Ca^{2+} in the longitudinal smooth muscle spontaneous activity from sheep duodenum. The amplitude of motility was inhibited in Ca^{2+}-free medium. When the preparations were incubated in Ca^{2+}-free solution plus 2 mM EDTA, the spontaneous activity was nearly blocked. The motor activity in guinea-pig taenia coli was blocked in Ca^{2+}-free solutions or in the absence of extracellular calcium plus EDTA or EGTA (Casteels & Raeymaekers 1979). Furthermore, the magnitude of contraction against several agonists is similar in the two muscle cell types, but the sources of Ca^{2+} are different (Grider & Makhlouf 1988). Those authors proposed that in the circular muscle cells, the sources of Ca^{2+} responsible for agonist-induced contraction are intracellular and that the contraction was not affected by withdrawal of Ca^{2+} from the medium. However, in longitudinal

muscle cells, the contraction was mediated by Ca^{2+} influx and abolished in Ca^{2+}-free media plus 2 mM EGTA.

Our results suggest that the spontaneous activity is partly the result of an influx of Ca^{2+} which is not easily removed by a Ca^{2+}-free solution, but is by 2 mM EDTA. These results are in accordance with those obtained in noradrenaline-induced sustained contractions of rabbit aorta (Karaki et al 1984).

Verapamil, diltiazem and nifedipine, Ca^{2+}-channel antagonists, either decreased or blocked the phasic activity in sheep duodenum. Nifedipine was found to be 30000-fold more potent than verapamil and 60000-fold more potent than diltiazem in sheep duodenum motility. This is consistent with other reports where nifedipine was more potent than verapamil to reduce the pendular movement in rabbit ileum (Beleslin et al 1985), the spontaneous activity in rabbit duodenum (Coruzzi & Poli 1987), K^+ contractions in canine and primate isolated colonic muscle (Barone et al 1986), and in peristaltic reflex and propulsion activity in guinea-pig colon (Lecchini et al 1991). Furthermore, verapamil and diltiazem inhibited the pendular movement (Beleslin et al 1985), the Ca^{2+} inward current (Terada et al 1987), the muscular activity (Lecchini et al 1991) and the duodenal contractility (Coruzzi & Poli 1987).

The present results are in agreement with those obtained in previous research in which nifedipine is found to be more efficient than verapamil and diltiazem in decreasing pressure or contractile amplitude (Traube & McCallum 1984) and also where verapamil and nifedipine antagonized carbachol-induced tonic contractions in rat duodenum and proximal colon (Maggi et al 1985).

Trifluoperazine, a calmodulin antagonist, and TMB-8, an inhibitor of intracellular Ca^{2+} release, reduced the spontaneous activity from sheep duodenum, but required a higher concentration than that of nifedipine, verapamil or diltiazem. This suggests that the Ca^{2+}-channel blockers are more important inhibitors in the sheep duodenum activity than Ca^{2+} antagonists, which act at an intracellular level. Trifluoperazine reduced acetylcholine contractions in rabbit duodenum (Matthijs et al 1988) and in sheep duodenum (De Pedro et al 1993), and decreased the contractions produced by field electrical stimulation in rat duodenum (Coruzzi & Poli 1987). TMB-8 blocked the efflux of Ca^{2+} from intracellular stores without affecting influx due to which it has been used in several cell types to determine the role of intracellular stored calcium in cell activation (Chiou & Malagodi 1975; Ogawa & Ono 1988).

In conclusion, our results suggest that in the spontaneous motility in sheep duodenum, extracellular Ca^{2+} has a more important role than that of intracellular Ca^{2+}, and that Ca^{2+}-channel blockers are more sensitive in inhibiting the duodenal activity than Ca^{2+}-intracellular blockers.

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References

Arruebo, M. P., Alcalde, A. I., Murillo, M. D. (1987a) Mucosal-free ruminal circular smooth muscle response to transmural electrical stimulation. J. Vet. Pharmacol. Ther. 10: 349–350

Arruebo, M. P., Alcalde, A. I., Murillo, M. D. (1987b) Papel del calcio sobre la contracción inducida por potasio y noradrenalina en rumen de oveja. Rev. Esp. Fisiol. 43: 365–370

Barone, F. C., White, R. F., Ormsbee, H. S., Wasserman, M. A. (1986) Effects of calcium channel entry blockers, nifedipine and nilvadipine, on colonic motor activity. J. Pharmacol. Exp. Ther. 237: 99–106

Beleslin, D. B., Samardžić, R., Terzić, B., Jovanović-Mićić, D. (1985) The effect of nifedipine and verapamil on the pendular movements of the rabbit isolated ileum. J. Pharm. Pharmacol. 37: 276–277

Bolton, T. B. (1979) Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59: 606–718

Casteels, R., Raeymaekers, L. (1979) The action of acetylcholine and catecholamines on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. J. Physiol. 294: 51–68

Chiou, C. Y., Malagodi, M. H. (1975) Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino) octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Br. J. Pharmacol. 53: 279–285

Coruzzi, G., Poli, E. (1987) Changes in duodenal contractility induced by "calcium antagonists" with different modes of action. Gen. Pharmacol. 18: 69–74

De Pedro, M. J., Plaza, M. A., Arruebo, M. P., Murillo, M. D. (1993) Effect of calcium antagonists on acetylcholine-induced contractions in longitudinal smooth muscle strips from sheep duodenum. J. Gastrointest. Mot. 5: 17–22

Godfraind, T., Miller, R. C., Wibo, M. (1986) Calcium antagonism and calcium entry blockade. Pharmacol. Rev. 38: 321–416.

Grider, J. R., Makhlouf, G. M. (1988) Contraction mediated by Ca^{++} release in circular and Ca^{++} influx in longitudinal intestinal muscle cells. J. Pharmacol. Exp. Ther. 244: 432–437.

Karaki, H., Weiss, G. B. (1984) Calcium channels in smooth muscle. Gastroenterology 87: 960–970

Karaki, H., Weiss, G. B. (1988) Minireview: calcium release in smooth muscle. Life Sci. 42: 111–122

Karaki, H., Nakagawa, H., Urakawa, N. (1984) Comparative effects of verapamil and sodium nitroprusside on contraction and ^{45}Ca uptake in the smooth muscle of rabbit aorta, rat aorta and guinea-pig taenia coli. Br. J. Pharmacol. 81: 393–400

Lecchini, S., Marcoli, M., De Ponti, F., Castelletti, C. A., Frigo, G. M. (1991) Selectivity of Ca^{2+} channel blockers in inhibiting muscular and nerve activities in isolated colon. Br. J. Pharmacol. 102: 735–741

Maggi, C. A., Manzini, S., Meli, A. (1985) Regional selectivity of calcium blockers at intestinal level. Arch. Int. Pharmacodyn. 276: 202–221

Marin, J. (1988) Vascular effects of calcium antagonists. Uses in some cerebrovascular disorders. Gen. Pharmacol. 19: 295–306

Matthijs, G., Peeters, T. L., Vantrappen, G. (1988) Effect of different calcium modulators on motilin induced contractions of the rabbit duodenum. Comparison with acetylcholine. Regul. Pept. 21: 321–330

Milanov, M. P., Stoyanov, I. N., Boev, K. K. (1984) Electromechanical coupling in the complex stomach smooth muscles. Gen. Pharmacol. 15: 99–105

Ogawa, N., Ono, H. (1988) Effect of 8-(*N*,*N*-diethylamino)-octyl-3,4,5 trimethoxybenzoate(TMB-8), an inhibitor of intracellular Ca^{2+} release, on autoregulation of renal blood flow in the dog. Naunyn Schmiedebergs Arch. Pharmacol. 338: 293–296

Parekh, A. B., Brading, A. F. (1991) The sources of calcium for carbachol-induced contraction in the circular smooth muscle of guinea-pig stomach. Br. J. Pharmacol. 104: 412–418

Snape, W. J. (1982) Effect of calcium on neurohumoral stimulation of feline colonic smooth muscle. Am. J. Physiol. 243: G134–G140

Spedding, M., Paoletti, R. (1992) III Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacol. Rev. 44: 363–376

Terada, K., Kitamura, K., Kuriyama, H. (1987) Blocking actions of Ca^{2+} antagonists on the Ca^{2+} channel in the smooth muscle cell membrane of rabbit small intestine. Pflügers Arch. 408: 552–557

Traube, M., McCallum, R. W. (1984) Calcium-channel blockers and the gastrointestinal tract. Am. J. Gastroenterol. 79: 892–896

Yu, J., Bose, R. (1991) Calcium channels in smooth muscle. Gastroenterology 100: 1448–1460