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Evaluation of contraction time and recovery period as a parameter in the calcium antagonistic action on the K^+-depolarized rat duodenum

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Abstract—Verapamil, nifedipine, phentolamine, tolazoline, gentamicin and neomycin inhibited calcium-induced contractions of K^+-depolarized duodenum of the rat by shifting the concentration-response curves to the right. Non-competitive inhibitions were observed with trifluoperazine, lidoflazine, procaine and tetracaine. Lanthanum behaved as a partial agonist in this preparation, while nitroprusside was ineffective. Contraction times in the presence of the antagonists and recovery time of the Ca^{2+} responses after the removal of the antagonists from the bathing medium were evaluated. From the findings, it is suggested that the contraction time and the time required for tissue recovery after removal of a Ca^{2+} antagonist are parameters making K^+-depolarized rat duodenum a potential tool for the evaluation of the pharmacological effects of Ca^{2+} antagonists.

Recent studies have strongly suggested that two distinct types of Ca^{2+}-channel, voltage-dependent Ca^{2+} and receptor-operated Ca^{2+} exist in smooth muscle (Weiss 1983; Karaki & Weiss 1984). Furthermore, it has been proposed that three functional classes for the antagonists of voltage-dependent Ca^{2+} channels exist with appropriate binding sites (Spedding 1985). Accordingly, dihydropyridines, like nifedipine, have been accepted as type-I Ca^{2+} antagonists. Diphenylalkylamines including trifluoperazine and lidoflazine have been classified as type-III Ca^{2+} antagonists. The other main Ca^{2+} antagonists, diltiazem and verapamil, have been considered as type-II antagonists.

Procaine and tetracaine exert a Ca^{2+} antagonistic action as a consequence of membrane stabilization (Bowman & Rand 1980; Burdyga & Magura 1986). Therefore, their effect may be considered non-specific. Furthermore, some α-adrenoceptor blocking agents, such as phentolamine are able to block Ca^{2+} influx via receptor-operated Ca^{2+} channels (Awad et al 1983; Kazda et al 1983). Sodium nitroprusside is another blocker of receptor operated Ca^{2+} channels, although it is ineffective on voltage-dependent Ca^{2+} channels (Karaki & Weiss 1984).

Lanthanum is a Ca^{2+} channel blocker which is also able to inhibit superficial Ca^{2+}-binding sites as well as voltage-dependent Ca^{2+} channels. It is reported to behave as a partial agonist of Ca^{2+} on aortic smooth muscle (Weiss 1974, 1983; Weiss & Goodman 1975). Most probably, its effect at low doses depends on enhanced Ca^{2+} uptake (Weiss 1983). Certain aminoglycoside antibiotics have also been found to inhibit superficial binding sites of Ca^{2+} (Goodman et al 1974; Goodman & Adams 1976).

There are complications in the classification of Ca^{2+} channels in smooth muscles resulting from the chemical structures of the antagonists and/or variation in the mode of actions. In this study, we aimed to investigate the action of the above compounds on the K^+-depolarized duodenum of the rat in order to evaluate their differential effects.

Materials and methods

The method for determining the response to Ca^{2+} of the K^+-depolarized duodenum of the rat was similar to that used with guinea-pig taenia coli (Ferrari & Carpenedo 1968a; Simonis et al 1971) except that calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$) was used as the spasmogenic agent to avoid auto-inhibitory effects associated with calcium chloride (Ferrari & Carpenedo 1968b; Öztürk et al 1987).

To record the Ca^{2+}-induced contractions, the rat proximal duodenum mounted in KNO_3-Ringer was connected to an isotonic transducer (Ugo Basile, No. 7006) coupled to a recorder (Ugo Basile, No. 7050), the load on the tissue was 0·50 g.

Non-cumulative dose-response curves to Ca^{2+} (3×10^{-6}–$9{\cdot}6 \times 10^{-5}$ M) were obtained in the absence and presence of the antagonists: nifedipine (Bayer), verapamil (Knoll), trifluoperazine (Sigma), lidoflazine (Sigma), procaine (Hoechst), tetracaine (Sigma), phentolamine (Ciba), tolazoline (Ciba), sodium nitroprusside (Aldrich), lanthanum chloride (Aldrich), neomycin (Sigma), gentamicin (Sigma). The preparation was incubated with one concentration of a drug for 15 min after which the dose-response procedure was repeated. All dilutions, except those of nifedipine, were prepared in water and administered into the bathing medium in a volume not exceeding 0·01 mL. Nifedipine was dissolved in water-ethanol (99:1) and added to the organ bath in a volume of 0·005 mL. The solvent in the same volume did not affect Ca^{2+}-induced contractions.

Competitive (pA_2) and non-competitive (pD'_2) antagonist affinity constants were calculated in order to evaluate action of the antagonists on the K^+-depolarized rat duodenum (Ariens & Van Rossum 1957; Arunlakshana & Schild 1959). Schild's slopes were calculated by plotting $-\log$ (drug concentration) against log (Ca^{2+} concentration ratio -1). The time required for maximal contraction to be attained before and after each antagonist was determined.

Recovery time was estimated after removal of each antagonist at the experimentally determined concentration that caused approximately 50% inhibition of the Ca^{2+}-induced contractions. The tissue was washed with approximately 10 mL of Ca^{2+}-free Tyrode solution 6 times at 5 min intervals. Then, every 5 min, tissue responses to a constant Ca^{2+} concentration ($4{\cdot}8 \times 10^{-5}$ M) were checked in the KNO_3-Ringer medium and compared with the initial Ca^{2+} responses in the absence of the antagonists.

Values reported are the mean ± s.e.m. of the experiments. Where indicated, the significance of the difference between the mean values was determined by Student's *t*-test and regression analysis was applied to examine the parallelism between the non-cumulative dose-response curves obtained in the absence and presence of the antagonists (Finney 1978).

Results

Nifedipine, verapamil, phentolamine, tolazoline, gentamicin and neomycin inhibited the Ca^{2+}-induced contractions of the K^+-depolarized rat duodenum, shifting the concentration-response curves to the right (Fig. 1). Because such a shift on response curve for agonist vs antagonist does not provide complete proof of competitive antagonism (Arunlakshana &

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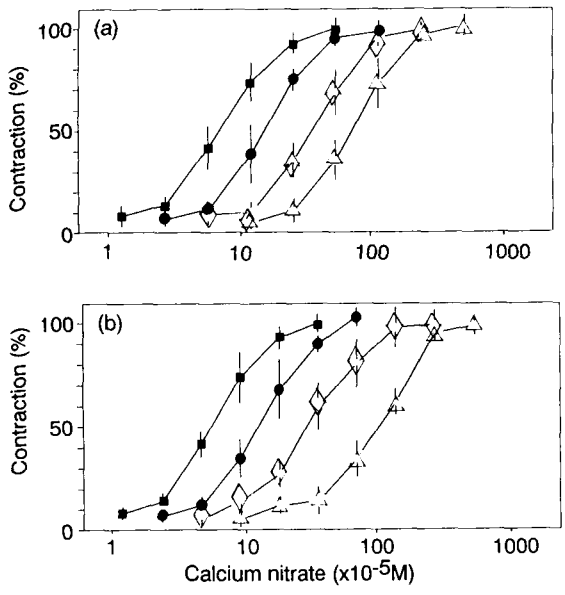

FIG. 1. Inhibitory effect of agents on the Ca^{2+}-induced contractions of K^+-depolarized rat duodenum (a) control (■——■, n = 15) and in the presence of $1{\cdot}6 \times 10^{-10}$ M verapamil (●——●, n = 5), $1{\cdot}8 \times 10^{-6}$ M phentolamine (◇——◇, n = 7), $3{\cdot}2 \times 10^{-6}$ M tolazoline (△——△, n = 8); (b) control (■——■, n = 15) and in the presence of $1{\cdot}4 \times 10^{-10}$ M nifedipine (●——●, n = 6), 7×10^{-6} M neomycin (◇——◇, n = 6), $1{\cdot}4 \times 10^{-4}$ M gentamicin (△——△, n = 6). Vertical bars indicate ± s.e.m.

Schild 1959), the slopes of the Schild plots for the above drugs were calculated and found to differ from unity (Table 1).

Trifluoperazine, lidoflazine, tetracaine and procaine inhibited the contractions elicited by Ca^{2+} in a non-competitive manner. Sodium nitroprusside was ineffective on the Ca^{2+}-induced contractions. The antagonists alone, except of lanthanum, had no effect on the K^+-depolarized rat duodenum. $7{\cdot}5 \times 10^{-7}$ to $1{\cdot}2 \times 10^{-5}$ M lanthanum, caused contraction of the depolarized duodenum, that was not dose-dependent and showed tachyphylaxis. This response was enhanced in the presence of a sub-contractile concentration of Ca^{2+} ($3{\cdot}8 \times 10^{-7}$ M) and abolished

Table 1. pA_2 and pD'_2 values for the Ca^+-antagonists as measured on the K^+-depolarized duodenum of the rat.

Antagonists	pD'_2	pA_2
Nifedipine (n = 6)	n.a.†	8·86 ± 0·02 a = −0·33 ± 0·02‡
Verapamil (n = 5)	n.a.	8·64 ± 0·01 a = −0·42 ± 0·03
Phentolamine (n = 7)	n.a.	5·63 ± 0·02 a = −1·20 ± 0·01
Tolazoline (n = 8)	n.a.	5·32 ± 0·03 a = −1·18 ± 0·07
Gentamicin (n = 6)	n.a.	4·85 ± 0·06 a = −0·57 ± 0·01
Neomycin (n = 5)	n.a.	5·15 ± 0·03 a = −0·79 ± 0·25
Trifluoperazine (n = 8)	6·98 ± 0·04	n.a.
Lidoflazine (n = 7)	6·83 ± 0·06	n.a.
Tetracaine (n = 5)	3·79 ± 0·02	n.a.
Procaine (n = 6)	2·79 ± 0·04	n.a.
Lanthanum (n = 8)	4·62 ± 0·05	n.a.
Nitroprusside (n = 5)	< 1·85*	< 1·85

† Non-applicable. ‡ Slope of Schild plot. * Ineffective.

in the presence of $1{\cdot}4 \times 10^{-11}$ M nifedipine (Fig. 2). Higher concentrations of lanthanum ($> 2{\cdot}4 \times 10^{-5}$ M) inhibited Ca^{2+} (3×10^{-6} to $9{\cdot}6 \times 10^{-5}$ M)-induced contractions, non-competitively. Table 1 shows apparent pA_2 and pD'_2 values from these studies.

Of the drugs tested, only phentolamine, tolazoline, trifluoperazine and lidoflazine delayed the attainment of maximal contraction induced by $4{\cdot}8 \times 10^{-4}$ M Ca^{2+} (Table 2). Table 3 shows the recovery times after removal of the antagonists.

Discussion

In this study, it was observed that nifedipine ($1{\cdot}4 \times 10^{-11}$–$1{\cdot}4 \times 10^{-9}$ M) and verapamil ($1{\cdot}6 \times 10^{-10}$–$1{\cdot}6 \times 10^{-8}$ M) inhibited the Ca^{2+}-induced contractions of the depolarized duodenum. However, the slopes of Schild plots were less than unity, indicating the antagonism is not purely competitive. On the other hand, the recovery period after nifedipine and verapamil was shorter than that after the other antagonists tested suggesting that the Ca^{2+}-antagonistic actions of verapamil and nifedipine are specific.

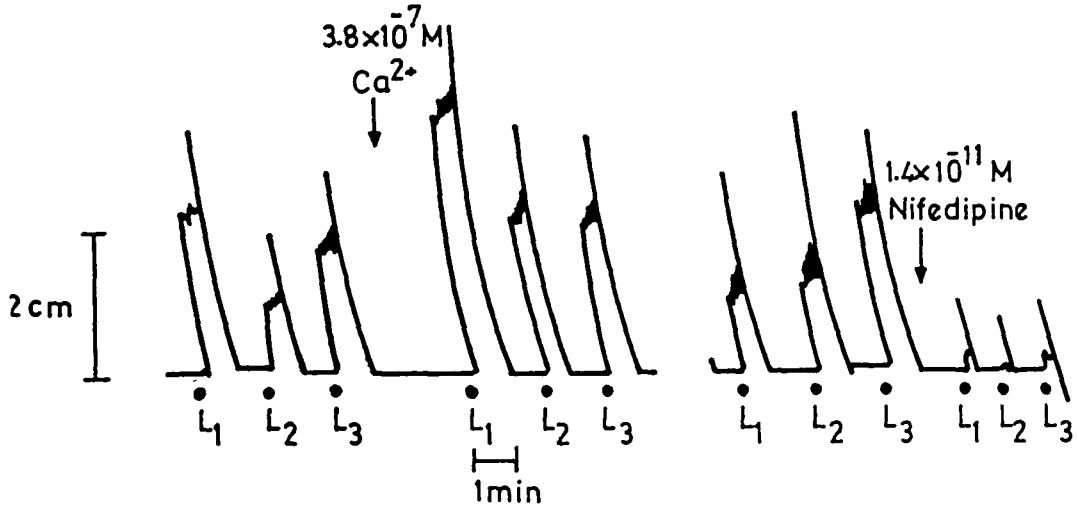

FIG. 2. Contractile effect of lanthanum on K^+-depolarized rat duodenum. Lanthanum doses applied into the organ bath; L_1: $7{\cdot}5 \times 10^{-7}$ M, L_2: $3{\cdot}0 \times 10^{-6}$ M, L_3: $1{\cdot}2 \times 10^{-5}$ M.

Table 2. Effect of the antagonists on the contraction time of the K^+-depolarized duodenum of the rat.

Treatment	Time (s)
None (n = 15)	36·60 ± 4·31
+ Nifedipine (n = 6)	40·21 ± 3·99
+ Verapamil (n = 5)	37·80 ± 5·20
+ Phentolamine (n = 7)	47·25 ± 3·28*
+ Tolazoline (n = 8)	46·12 ± 2·98*
+ Gentamicin (n = 6)	35·88 ± 3·82
+ Neomycin (n = 5)	36·42 ± 2·99
+ Trifluoperazine (n = 8)	52·32 ± 5·42†
+ Lidoflazine (n = 7)	46·23 ± 4·12*
+ Tetracaine (n = 5)	38·23 ± 2·23
+ Procaine (n = 6)	39·83 ± 3·35
+ Lanthanum (n = 8)	34·83 ± 4·29
+ Nitroprusside (n = 5)	40·23 ± 5·82

* $P < 0.05$, † $P < 0.01$ relative to control.

Table 3. Recovery time after antagonists.

Antagonist	Recovery time (min)
Trifluoperazine (n = 8)	68·68 ± 4·21
Lidoflazine (n = 7)	66·78 ± 7·50
Lanthanum (n = 8)	45·99 ± 6·39
Procaine (n = 6)	41·78 ± 8·60
Tetracaine (n = 5)	40·09 ± 5·20
Tolazoline (n = 8)	37·13 ± 6·03
Neomycin (n = 5)	37·10 ± 5·80
Gentamicin (n = 6)	36·52 ± 4·23
Phentolamine (n = 7)	35·23 ± 5·11
Verapamil (n = 5)	30·52 ± 6·45
Nifedipine (n = 6)	28·64 ± 8·23
Nitroprusside (n = 5)	n.a.

n.a. = not applicable

Trifluoperazine ($3{\cdot}9 \times 10^{-8}$–$6{\cdot}25 \times 10^{-7}$ M) and lidoflazine ($7{\cdot}6 \times 10^{-9}$–$7{\cdot}6 \times 10^{-7}$ M) were non-competitive Ca^{2+} antagonists and the time required for the recovery of maximal contraction after their wash out was longer than that of the other drugs tested. The cross-bridge cycling rate which is essential for contraction is related to the levels of calmodulin-dependent myosin phosphorylation during maintained contractions of smooth muscles (Aksoy et al 1982; Murphy et al 1983). So, calmodulin inhibition may be implicated in the actions of trifluoperazine and lidoflazine on the duodenum preparation.

While Ca^{2+}-induced contractions of the K^+-depolarized duodenum were not affected by sodium nitroprusside, an inhibitor of receptor-operated Ca^+ channels, they were inhibited by phentolamine and tolazoline which are also inhibitors of Ca^{2+} channels via α-adrenoceptors. However, the concentrations we used are higher than those required for α-adrenoceptor blockade. This suggests that this inhibition is not due to blockade of receptor-operated Ca^{2+} channels. Kazda et al (1983) observed that phentolamine at the concentration required for α-adrenoceptor blockade is ineffective on voltage-dependent Ca^{2+} channels. On the other hand, it seems likely that the inhibitory actions of phentolamine and tolazoline on the depolarized duodenum could be a consequence of blockade of voltage-dependent Ca^{2+} channels.

Gentamicin and neomycin were also found to be inhibitors of Ca^{2+} on the K^+-depolarized duodenum, but the slopes of their Schild plots were also not unity suggesting different sites of action. Inhibition most probably results from the blockade of voltage-dependent Ca^{2+} channels since the contraction time was not affected in their presence. It has been shown that aminoglycoside antibiotics do not enter the cell in significant quantities (Goodman 1978) and their Ca^{2+} antagonistic action could thus be attributed to binding at superficially located sites and a subsequent inhibition of Ca^{2+} uptake.

Local anaesthetics are believed to induce cell membrane stabilization and thus prevent the generation and conduction of nerve impulse (Bowman & Rand 1980; Narahashi & Frazier 1971). Therefore, they may inhibit sodium and potassium channels as well as Ca^{2+} channels (Ritchie 1975). In our experiments, tetracaine and procaine non-competitively inhibited the Ca^{2+}-induced contractions of the K^+-depolarized duodenum suggesting a non-specific mechanism. The contraction time did not change after either anaesthetic suggesting an absence of action on the contractile proteins in the smooth muscle. In addition, we observed that the recovery periods after their wash out were longer than those for other drugs.

According to Weiss (1983) lanthanum is able to inhibit almost all kinds of Ca^{2+} channels and enhanced Ca^{2+} uptake has been observed after low doses (Weiss 1974; Weiss & Goodman 1975). We observed that it caused contraction of the K^+-depolarized duodenum in a non-concentration-dependent manner and at low doses. As the contraction was inhibited by nifedipine and enhanced by sub-contractile Ca^{2+} concentrations, it seems likely that low doses of lanthanum enhance Ca^{2+} uptake in the K^+-depolarized duodenum.

In conclusion, it is suggested that the contraction time and the time required for tissue recovery after removal of Ca^{2+} antagonist are useful parameters in the evaluation of their effects on the K^+-depolarized duodenum of the rat which may be considered as a potential tool in the evaluation of the pharmacological effects of Ca^{2+} antagonists.

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Effect of myrcene on nociception in mice

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Abstract—Myrcene, a monoterpene isolated from lemon grass oil (*Cymbopogon citratus*) has been investigated for antinociception in mice by a low temperature (51·5 ± 0·5°C) hot plate method and by the acetic acid-induced writhing test. Significant inhibition of nociception was seen in the tests with myrcene at doses of 10 and 20 mg kg^{-1} (i.p.) or at 20 and 40 mg kg^{-1} (s.c.), respectively. The antinociceptive effect was significantly antagonized by naloxone (1 mg kg^{-1}) or yohimbine (2 mg kg^{-1}). The results suggest that myrcene is capable of inducing antinociception in mice, probably mediated by α_2-adrenoceptor stimulated release of endogenous opioids.

Essential oil from *Cymbopogon citratus* (D.C) Stapf (Graminеae), commonly known as lemon grass oil, is used as a folk remedy in Brazil and elsewhere for the treatment of gastrointestinal disturbances (Alves et al 1960). The reported major constituents are citral (80%), an aldehyde, and myrcene (16%), a monoterpene (Silva & Bauer 1971). Despite its widespread use no reports on the pharmacological activity of this oil or its major constituents appear to have been published. In this study we show that the constituent myrcene exerts an antinociceptive effect in the mouse.

Materials and methods

Swiss male mice, 25–28 g, were kept at an ambient temperature of 25 ± 2°C and fed on standard pellet chow with water freely available.

Antinociceptive activity. This was measured by a low temperature (51·5 ± 0·5°C) hot plate method of Eddy & Leimbach (1953) and by the acetic acid-induced writhing test of Koster et al (1959).

In the hot plate test, each mouse received two trials on the hot plate, separated by a 30 min interval. The first trial familiarized the animal with the test procedure and the second trial served as the control reaction time (licking of hind feet or jumping) for the animal. Mice were preselected, any showing a reaction time greater than 10 s were not used. Immediately after the second trial, groups of mice (10 per group) were given myrcene (10 or 20 mg kg^{-1}) or vehicle (0·9% NaCl solution containing 2% Tween 80) intraperitoneally (i.p.) in a volume of 10 mL kg^{-1}. The reaction time for each mouse was determined on the hot plate surface at 15 min intervals after drug administration for a total of 120 min. To avoid possible injury, there was a cut off period of 45 s while measuring the reaction time. In a few experiments, the influence of simultaneously administered naloxone (1 mg kg^{-1} s.c.) or yohimbine (2 mg kg^{-1} s.c.) on myrcene-induced antinociception was assessed.

In the acetic acid test, each mouse was injected i.p. with 0·6% (v/v) aqueous acetic acid (10 mL kg^{-1}) 30 min after s.c. administration of myrcene (20 or 40 mg kg^{-1}) or vehicle. Groups of six to eight animals were used. Five minutes after acetic acid treatment the mice were observed for 20 min and the number of writhes counted. The effect of simultaneous administration of naloxone (1 mg kg^{-1} s.c.) or yohimbine (2 mg kg^{-1} s.c.) was also verified on the nociceptive activity of 20 mg kg^{-1} myrcene.

Drugs used were: myrcene (CEPEQ, Brazil), morphine (CEME, Brazil), naloxone (Sigma) and yohimbine (Sigma). Myrcene was suspended in 0·9% NaCl solution containing 2% Tween 80. All other drugs were diluted in 0·9% NaCl solution. The data were evaluated by use of ANOVA and *t*-independent test. Statistical significance was assigned for $P < 0·05$.

Results

Results of the hot plate test are shown in Fig. 1. Myrcene (10 or 20 mg kg^{-1}) administered i.p., produced a dose related elevation in the reaction time lasting about 120 min with a peak effect between 45 and 60 min. The antinociceptive response, however, was inferior to that produced by morphine (5 mg kg^{-1}, i.p.). Myrcene (20 mg kg^{-1})-induced analgesia was partially atte-

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