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Effects of the Calcium Antagonists Diltiazem, Verapamil and Nitrendipine on the Contractile Responses of Guinea-pig Isolated Ileum to Electrical Stimulation or Carbachol

D. STANEVA-STOYTCHEVA AND K. VENKOVA

Institute of Physiology, Bulgarian Academy of Sciences, 1113 Acad. G. Bonchev str. bl. 23, Sofia, Bulgaria

Abstract—The effects of the organic Ca^{2+} antagonists nitrendipine, verapamil and diltiazem on the cholinergic contractile responses induced by field electrical stimulation or carbachol (0·1 μM) and on contractions evoked by high concentration KCl (30 mM) were studied in isolated preparations from the guinea-pig ileum. The three Ca^{2+} antagonists dose-dependently suppressed the contractile responses showing the same order of potency (nitrendipine > verapamil > diltiazem) with the three different types of stimulation. Comparison of the IC50 values of the Ca^{2+} antagonists for carbachol-, KCl- and electrically-evoked contractions demonstrated that the carbachol-evoked contractions were most sensitive to the inhibitory action of the antagonists tested. The presynaptic inhibitory effect of (Met)enkephalin (10 nM) on the electrically-evoked cholinergic contractions was only slightly potentiated by high concentrations (1 or 10 μM) of nitrendipine and diltiazem and remained unchanged by verapamil. The results suggest that the Ca^{2+} antagonists tested block mainly the carbachol-activated L-type Ca^{2+} channels on the smooth muscle cells, while the effects on the N-type Ca^{2+} channels are insignificant, except for the high concentrations of nitrendipine and diltiazem.

The inhibitory effect of organic Ca^{2+} antagonists on the electro-mechanical coupling in the smooth muscle cells has been shown to be related to the inhibition of the potential-dependent influx of Ca^{2+} (Bolton 1979). However, the structural heterogeneity of the different organic Ca^{2+} antagonists may result in considerable differences in their pharmacological and clinical effects (Henry 1980; Nayler 1980; Braunwald 1982). That acetylcholine liberation is calcium-dependent has long been known (Katz & Miledi 1968). The intracellular concentration of Ca^{2+} in the neuron is considered to be responsible for the coupling of the stimulus to neurotransmitter secretion. It is generally accepted that the smooth muscle contraction occurring after acetylcholine is induced mainly by the release of Ca^{2+} from intracellular stores (Edman & Schild 1962). The same mechanism has also been described for carbachol-induced contractions, but according to Brading & Sneddon (1980) the carbachol-sensitive intracellular Ca^{2+} stores are rapidly depleted when extracellular Ca^{2+} is omitted. There are also data that in guinea-pig taenia coli the carbachol-induced contractions are less sensitive to extracellular Ca^{2+} compared with the acetylcholine-induced contractions (Mitchelson & Ziegler 1984). The differences have been explained as due to the different interactions of acetylcholine and carbachol with muscarinic cholinoceptors.

The aim of the present study was to establish whether there are differences between the inhibitory effects of the organic Ca^{2+} antagonists on the contractile responses induced by electrical field stimulation or by direct activation of post-synaptic muscarinic cholinoceptors with carbachol. The guinea-pig isolated ileum was used as a suitable model for studies of cholinergic smooth muscle responses. The role of extracellular Ca^{2+} and the Ca^{2+} movements through the smooth muscle membrane during the development of contractile responses to muscarinic agonists or high K^+ have been studied in detail in the ileal longitudinal smooth muscle (Chang & Triggle 1973; Rosenberger et al 1979; Bolger et al 1983). Two phases, which have different sensitivity to extracellular Ca^{2+} are now defined in the responses of the ileum to muscarinic agonists and high K^+ (Triggle et al 1989). The inhibitory potency of three compounds, which are widely used in the therapy of cardiovascular disorders and are representatives of the main groups of organic Ca^{2+} antagonists, was tested against the twitch-like cholinergic responses to electrical stimulation and the first phasic component of the responses to carbachol. Nitrendipine (a dihydropyridine), verapamil (a phenylalkylamine) and diltiazem (a benzothiazepine) were used in the experiments. In order to examine the mechanism of action of the three Ca^{2+} antagonists, we also studied their effects on the phasic contractile responses evoked by high KCl and on the (Met)enkephalin-induced inhibition of electrically-evoked twitch-like contractile responses.

Correspondence: D. Staneva-Stoytcheva, Department of Experimental Pharmacology, Institute of Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str. bl. 23, 1113 Sofia, Bulgaria.

Materials and Methods

Smooth muscle preparations

Male guinea-pigs, 250–300 g, were killed by a blow on the neck and were exsanguinated by cutting the carotid arteries. The non-terminal part of the ileum (about 15 cm proximal to the ileocaecal sphincter) was removed and washed with Krebs solution at room temperature (21°C). Segments 20 mm long were cut out and mounted vertically in organ baths containing 10 mL Krebs solution bubbled with 95% O_2–5% CO_2 at 37°C. The contractile activity was recorded under

isometric conditions by means of a strain gauge (M 1000 B, Micritechna) on a recording device (Type-175, Kutesz). The preparations were initially suspended under 1 g tension. There was an equilibration period of 40–60 min before any measurements were made.

Electrical stimulation

Field electrical stimulation (FES) was applied through a pair of wire platinum electrodes vertically mounted into the organ bath (Paton & Zar 1968). Contractile responses of submaximal amplitude were elicited by single pulses of 0·4 ms duration and a constant voltage of 30 V generated at 15 s intervals. The electrical stimulation was provided by means of a pulse generator ESU-2 (Medexport).

Pharmacological analysis

The effects of the calcium antagonists on the contractile responses induced by electrical stimulation or by application of carbachol or KCl to the nutrient solution were tested in separate experiments. Carbachol was used at a concentration of 0·1 μM to induce contractile responses whose amplitude was comparable with the amplitude of the electrically-evoked contractions. High KCl-induced contractions of similar amplitude were evoked by increasing the concentration of KCl in the solution to 30 mM. The treatment with carbachol or KCl did not exceed 1 min, since the stimulant substances were washed out immediately after the achievement of the maximum of the phasic contractions.

The Ca^{2+} antagonists were added at increasing concentrations of 1 nM to 100 μM over 10 min. The concentration range and time were chosen after preliminary experiments, which had shown that each concentration of the antagonists tested produced maximal inhibition within the first 10 min of treatment. In the experiments on electrically-induced responses the Ca^{2+} antagonists were added in a cumulative manner. When studying the responses to carbachol or high KCl the latter were washed out with Krebs solution containing the Ca^{2+} antagonist tested.

The concentration-effect curves were subjected to linear regression analysis and IC50 values for the Ca^{2+} antagonists were calculated. The 95% confident limits were also presented to assess the statistical significance of the differences ($P<0·05$). The relative potency of the antagonists was estimated by comparison of the mean IC50 values.

When studying the action of the Ca^{2+} antagonists on the effects of (Met)enkephalin on the electrically-evoked contractions, (Met)enkephalin was added at a concentration of 10 nM to induce pronounced, but submaximal, inhibition. To avoid the postsynaptic influence of the Ca^{2+} antagonists, (Met)enkephalin effects were expressed as percentage of the twitch responses occurring in the presence of each concentration of the antagonist and taken as 100%. The differences were assessed for statistical significance using Student's *t*-test for paired data at $P<0·05$.

Solution and drugs

Modified Krebs solution consisted of (mM): NaCl 120, KCl 5·9, $NaHCO_3$ 15·4, NaH_2PO_4 1·2, $MgCl_2$ 1·2, $CaCl_2$ 2·5 and glucose 11·5.

Drugs used were: verapamil (Orion, Finland), nitrendipine (Sopharma, Bulgaria), diltiazem (Orion), carbamylcholine chloride (carbachol) (Sigma), (Met)enkephalin (Sigma), naloxone (Sigma) and atropine sulphate (Merck). All compounds except for nitrendipine were dissolved in distilled water and diluted to their final concentrations in Krebs solution. Nitrendipine was dissolved at a concentration of 1 mM in 70% ethanol, diluted in 30% ethanol and added to the organ bath at a constant volume of 0·01 mL. Under these conditions the concentration of ethanol in the nutrient solution had no effects on the activity of the preparations.

Results

Effects on electrically-evoked contractile responses

The isolated segments of the guinea-pig ileum responded to FES applied as single pulses (0·4 ms, 30 V) with twitch contractions of submaximal amplitude. Atropine (1 μM) completely abolished the electrically-evoked responses showing their cholinergic nature. When FES was applied at 15 s intervals the twitch amplitudes remained constant for longer than 90 min. The Ca^{2+} antagonists, added cumulatively in increasing concentrations of 0·1 nM to 100 μM, produced concentration-dependent reduction of the electrically-evoked responses. The latter were completely inhibited by the addition of nitrendipine or diltiazem at concentrations of 10 or 50 μM, respectively, while verapamil at the highest concentration (100 μM) markedly reduced, but failed to abolish, the contractions induced by FES. The mean regression lines obtained for each Ca^{2+} antagonist are presented in Fig. 1. The most pronounced effect was obtained with nitrendipine, which yielded the lowest IC50 value, followed by verapamil and diltiazem (Table 1). The IC50 values for nitrendipine, verapamil and diltiazem differed significantly ($P<0·05$) from one another. Potencies of verapamil and nitrendipine compared with diltiazem were 7·5 and 25, respectively.

Effects on carbachol-induced contractile responses

Carbachol (0·1 μM) elicited contractile responses of the ileum immediately after its application; a maxium was reached in the first minute of treatment. The responses were of submaximal amplitude and could be repeatedly induced after wash-

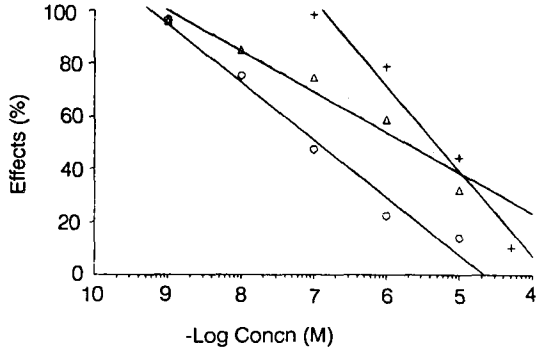

FIG. 1. Mean regression lines illustrating the inhibitory action of nitrendipine (○), verapamil (△) and diltiazem (+) on the contractile effect induced by FES (0·4 ms, 30 V) on guinea-pig isolated ileum. The effects of FES in untreated preparations were accepted to be 100%. Each point represents the mean value of at least 6 experiments on preparations isolated from different animals.

Table 1. IC50 Values of verapamil, nitrendipine and diltiazem defined against the contractile responses induced by FES (0·5 ms, 30 V), carbachol (0·1 μM) or KCl (30 mM) on guinea-pig ileum. Mean values (μM) obtained from 6 preparations from different animals as well as the 95% confident limits (given in parentheses) are presented.

	Responses to		
Ca-antagonist	Electrical stimulation	Carbachol	KCl
Nitrendipine	0·226 (0·137–0·315)	0·016 (0·009–0·023)	0·147 (0·090–0·204)
Verapamil	0·769 (0·578–0·960)	0·381 (0·176–0·586)	2·084 (1·360–2·808)
Diltiazem	5·646 (4·310–6·977)	2·801 (2·118–3·484)	5·223 (2·792–7·654)

ing. Concentration-effect curves were obtained for the Ca^{2+} antagonists following the procedure used with FES. The only difference was that after the contraction, carbachol was washed out with Krebs solution containing the Ca^{2+} antagonist tested. The effects of the Ca^{2+} antagonists showed a concentration-dependent decrease of the amplitude of the carbachol-induced contractions. The latter were completely abolished by nitrendipine or verapamil applied at concentrations of 0·1 and 50 μM, respectively. The effects of diltiazem were less pronounced and low-amplitude contractions were elicited by carbachol in the presence of diltiazem at a concentration of 100 μM. The regression lines illustrating the inhibition of carbachol-induced contractions are given in Fig. 2. The IC50 values calculated for nitrendipine, verapamil and diltiazem significantly ($P < 0·05$) differed from one another (Table 1). The relative potency (IC50) of verapamil compared with diltiazem was 7·3 and of nitrendipine 175, following the order of potency determined for the electrically-evoked contractions, i.e. nitrendipine > verapamil > diltiazem.

Effects on KCl-induced contractile responses

The effects of the Ca^{2+} antagonists on the contractile responses induced by increasing the concentration of potassium in the medium were also tested. When the concentration of KCl was increased from 5·9 to 30 mM the contractile responses of the ileum occurred immediately and could be repeatedly induced after washing. Concentration-effect curves for the action of the Ca^{2+} antagonists were obtained following the procedure described for carbachol-induced responses. Nitrendipine, verapamil or diltiazem applied at a low concentration (1 nM) slightly increased the amplitude of the contractile responses to KCl. With the higher concentrations, the Ca^{2+} antagonists decreased the amplitude of KCl-induced contractions in a concentration-dependent manner, the inhibitory effect being most pronounced with nitrendipine. The highest concentrations (10 μM) of the three Ca^{2+} antagonists almost completely abolished the contractions induced by KCl (Fig. 3). The IC50 values calculated for verapamil and diltiazem did not differ significantly ($P > 0·05$) from one another but each of them was significantly different ($P < 0·05$) from the IC50 value for nitrendipine (Table 1). When compared with diltiazem the relative potency of verapamil was 2·5 and of nitrendipine was 35.

Effects on the (Met)enkephalin-induced inhibition of electrically-evoked responses

(Met)enkephalin is known to suppress dose-dependently the electrically-evoked cholinergic contractions of guinea-pig isolated ileum (Paton 1957; Kosterlitz et al 1980). In our experiments the amplitude of the contractions evoked by FES (0·4 ms, 30 V) was reduced by 10 nM (Met)enkephalin. Under these conditions (Met)enkephalin exerted a constant effect inhibiting the contractile responses to about 75–80% of the control contractions. The preparations did not change their activity after washing out the enkephalin. The effects of

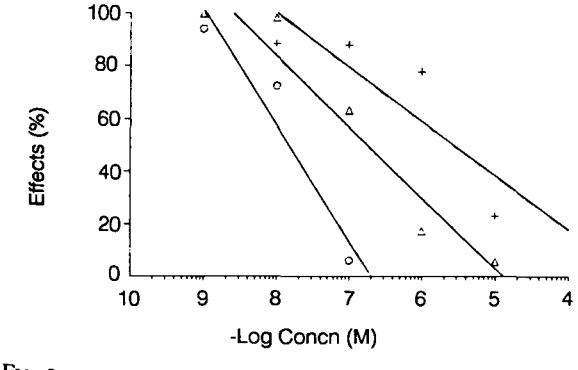

FIG. 2. Mean regression lines illustrating the inhibitory action of nitrendipine (○), verapamil (△) and diltiazem (+) on the contractile effect induced by carbachol (0·1 μM) on guinea-pig isolated ileum. The effects of carbachol in untreated preparations were accepted to be 100%. Each point represents the mean value of at least 6 experiments on preparations isolated from different animals.

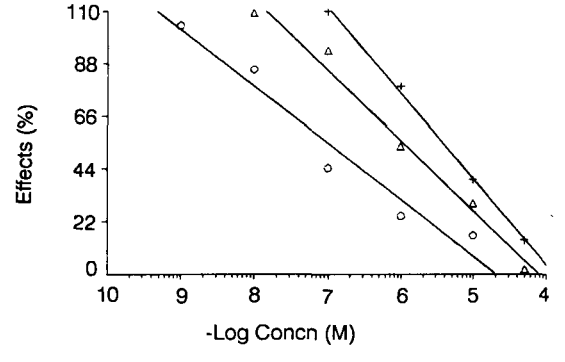

FIG. 3. Mean regression lines illustrating the inhibitory action of nitrendipine (○), verapamil (△) and diltiazem (+) on the contractile effect induced by KCl (30 mM) on guinea-pig isolated ileum. The effects of KCl in untreated preparations were accepted to be 100%. Each point represents the mean value of at least 6 experiments on preparations isolated from different animals.

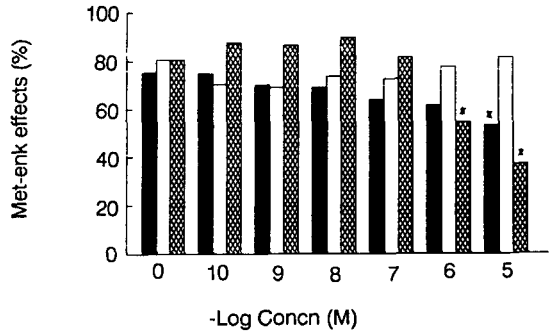

FIG. 4. Action of nitrendipine, verapamil and diltiazem on the effects of (Met)enkephalin (Met-enk, 10 nM) expressed as % of the contractile responses to FES (0·4 ms, 30 V) on guinea-pig isolated ileum. In three groups of experiments (Met)enkephalin effects are followed in untreated preparations (C) and after treatment with increasing concentrations of nitrendipine, verapamil and diltiazem. Student's *t*-test for paired data is used to access the statistical significance (* $P<0·05$) of the changes in (Met)enkephalin effect induced by nitrendipine, verapamil or diltiazem. Each bar represents the mean value of at least 6 experiments on preparations isolated from different animals. Diltiazem, solid bar; verapamil, open bar; nitrendipine, cross hatched bar.

(Met)enkephalin were reversibly blocked by naloxone (1 μM). Nitrendipine, verapamil and diltiazem were used at increasing concentrations of 0·1 nM to 10 μM. The effects of (Met)enkephalin (10 nM) were tested 10 min after application of each concentration of the Ca^{2+} antagonist. It was found that the inhibitory effect of (Met)enkephalin was not changed significantly ($P>0·05$) by the Ca^{2+} antagonists applied at concentrations increasing to 100 nM (Fig. 4). At low concentrations nitrendipine tended to decrease the inhibitory effect of (Met)enkephalin, while at the higher concentrations (1 and 10 μM) it significantly ($P<0·05$) potentiated the inhibition induced by (Met)enkephalin. Diltiazem also tended to potentiate the (Met)enkephalin effect but this potentiation was significant ($P<0·05$) only with the higher concentration of 10 μM. Unlike nitrendipine and diltiazem, verapamil, even at the highest concentration tested, did not change the effect of (Met)enkephalin despite its pronounced concentration-dependent inhibitory effects on the electrically-evoked contractions.

Discussion

These results demonstrate significant quantitative differences in the inhibitory effects of the Ca^{2+} antagonists nitrendipine, verapamil and diltiazem on the contractions of the guinea-pig isolated ileum evoked by FES, carbachol or KCl. With the three different types of stimulation the Ca^{2+} antagonists followed the same order of potency, i.e. nitrendipine > verapamil > diltiazem. These results are in agreement with literature values, reporting the same order of potency of the slow Ca^{2+} channels blocking agents in inhibiting KCl-induced contractions of intestinal smooth muscles (Godfraind et al 1986) and guinea-pig taenia coli (Mutafova-Jambolieva et al 1989).

Comparison of the IC50 values of nitrendipine, verapamil and diltiazem obtained with the different types of stimulation showed that the carbachol-induced contractions were most sensitive to the inhibitory action of the Ca^{2+} antagonists. Thus, the potency of nitrendipine was about 175 times that of diltiazem. Less sensitive to the action of the Ca^{2+} antagonists were the electrically-evoked contractions. The IC50 value of nitrendipine estimated for the electrically-evoked contractions was about 14 times higher than the IC50 value for carbachol-induced contractions. The ratio of IC50 of diltiazem for electrically-evoked contractions to that for carbachol-induced contractions was about 2. However, the IC50 values of verapamil for electrically-evoked and carbachol-induced contractions did not differ significantly. It is known that different types of Ca^{2+} channels are involved in neurotransmitter release and in the agonist-induced smooth muscle responses. According to recent publications, the release of acetylcholine from the cholinergic nerve terminals is associated mainly with the functions of N-type voltage-dependent Ca^{2+} channels, which play a pivotal role in the regulation of intracellular Ca^{2+} levels, necessary to induce neurotransmitter release from the neurons (Nowycky et al 1985; Reynolds et al 1986; Lundy & Frew 1988; Snyder & Wagner 1988). The N-type voltage-dependent Ca^{2+} channels are selectively blocked by ω-conotoxin (McCleskey et al 1986). Our results have demonstrated that the Ca^{2+} antagonists tested blocked mainly the carbachol-activated L-type Ca^{2+} channels of the smooth muscle cells, while the effects on the N-type Ca^{2+} channels were insignificant except for the highest concentrations of nitrendipine and diltiazem. Slight potentiation of the (Met)enkephalin-induced inhibition of the neurogenic responses to FES by the higher concentrations (1 or 10 μM) of nitrendipine or diltiazem was also observed. These findings are in accordance with the literature data (Triggle et al 1979; Kaplita & Triggle 1983) on the relatively high resistance of the N-type Ca^{2+} channels to the organic Ca^{2+} antagonists widely used in clinical practice. As for verapamil, the IC50 values for carbachol-induced and electrically-evoked responses were not significantly different, suggesting that the inhibitory effects of verapamil are predominantly, if not entirely, realized at the postsynaptic site of the neuromuscular junction. Moreover, the relative potency of verapamil was the same for electrically-evoked and carbachol-induced contractions. Even a high concentration of verapamil (10 μM) failed to change the presynaptic reduction of the electrically-evoked responses induced by (Met)enkephalin. Thus it is unlikely that there are verapamil-sensitive Ca^{2+} channels which could reduce the release of cholinergic neurotransmitter in the guinea-pig ileum.

Comparison of the IC50 values for carbachol- and KCl-induced contractions showed more potent inhibitory effects of the three Ca^{2+} antagonists on carbachol-induced responses. This supports the general view that carbachol and KCl could activate different Ca^{2+} channels—receptor-operated and voltage-dependent—and that the Ca^{2+} antagonists block mainly receptor-operated Ca^{2+} channels involved in the phasic responses to carbachol. The initial potentiation of the KCl-induced contractions by the lowest concentration (1 nM) of each of the Ca^{2+} antagonists might reflect the activation of some intracellular Ca^{2+} stores (Hurwitz & Joiner 1970; Van Breemen & Saida 1989).

The action of the Ca^{2+} antagonists may not only be by blockade of the Ca^{2+} channels. Karliner et al (1982) have reported that verapamil at concentrations of about 3 μM (which could be reached in clinical practice) competitively inhibit the binding of the muscarinic radiolabelled antago-

nist [^{3}H]QNB to muscarinic receptors. In an earlier work (Popova et al 1990) a reduction of the binding capacity of the cerebral cortical muscarinic receptors for [^{3}H]QNB was established after long-term treatment of rats with nifedipine or verapamil, which was more pronounced after nifedipine treatment. Some intracellular events, occurring after the agonist-receptor interaction, such as Ca^{2+} release from intracellular stores, inhibition of calmodulin activity and effects on the phosphoinositide system, which are involved in the carbachol-induced contraction could also be differently influenced by the Ca^{2+} antagonists (Gengo et al 1988; Van Rooijen & Traber 1986; Ek & Nahorski 1988). According to Johnson et al (1983) the verapamil analogue D-600 and diltiazem, at concentrations higher than 10 μM, could regulate the calmodulin-operating system resembling the action of the calmodulin antagonist W-7. In the rabbit ear artery diltiazem at concentrations exceeding 1 μM not only blocked the Ca^{2+} influx but also inhibited the release of Ca^{2+} from the intracellular stores (Saida & Van Breemen 1983).

The present results are of theoretical and practical interest because the different effects of the organic Ca^{2+} antagonists on the activity of the intestinal smooth muscle could mean the possible use of some of these drugs as spasmolytics (Samueli et al 1984).

Acknowledgement

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