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A novel benzothiazine Ca^{2+} channel antagonist, semotiadil, inhibits cardiac L-type Ca^{2+} currents

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Abstract

The influence of semotiadil fumarate, a novel vasoselective Ca^{2+} channel antagonist with a benzothiazine skeleton, was measured on the high-threshold Ca^{2+} current $I_{Ca,L}$ in guinea-pig ventricular myocytes prepared by coronary perfusion with collagenase solution. Patch- and voltage-clamp methods were used to measure $I_{Ca,L}$. Diltiazem, nifedipine and amlodipine were studied for comparison. Semotiadil could be shown to inhibit $I_{Ca,L}$ in a dose-dependent manner in concentrations similar to those of diltiazem but was less effective than amlodipine and nifedipine. The IC_{50} for nifedipine and amlodipine was in the range between 0.1 and 1 μM, and that of semotiadil and diltiazem was between 10 and 100 μM. Recovery from inactivation of $I_{Ca,L}$ in the control and under the influence of nifedipine (0.01 μM) and amlodipine (0.1 μM) was complete after 1 s. Semotiadil (0.1 μM) and diltiazem (1 μM) prolonged the time to full recovery to 20 s. This significant delay in the recovery of $I_{Ca,L}$ produced by semotiadil indicates a mode of action similar to that of the verapamil type of Ca^{2+} channel antagonists and makes a clear distinction between it and the dihydropyridines, which have no effect on the recovery process. The rate dependence of the effect in combination with a distinct influence of the holding potential underlines the use dependence of the mechanism underlying the effect of semotiadil. The well-known high vasoselectivity of semotiadil in combination with a relatively low Ca^{2+} channel antagonistic influence on the heart makes semotiadil an interesting candidate for the treatment of coronary heart diseases.

Keywords: Semotiadil; Diltiazem; Amlodipine; Nifedipine; Ca^{2+} current; Ventricular myocyte

1. Introduction

Semotiadil fumarate (SD-3211, (+)-(*R*)-2-[5-methoxy-2-[3-[methyl-[2-[(3,4-methylenedioxy)-phenoxy]ethyl]amino]propoxy]phenyl]-4-methyl-2*H*-1,4 benzothiazin-3(4*H*)-one hydrogen fumarate) is a novel vasoselective Ca^{2+} channel antagonist with a benzothiazine skeleton (Miyawaki et al., 1990; Nishimura et al., 1990; Yoneyama et al., 1990; Kageyama et al., 1991; Takada et al., 1991; Nakayama et al., 1992, 1994, Mori et al., 1995). This structure is different from that of all other Ca^{2+} channel antagonists, and semotiadil has been characterized as a predominant vasoselective drug with potent and long-lasting actions. Semotiadil was found to antagonize coronary artery contractions ten times more potently than diltiazem but to exert in guinea-pig atria only two-thirds of the negative inotropic effect of diltiazem (Nishimura et al., 1990). The dose that doubled coronary blood flow, prolonged AV conduction time by 15% and had to be increased 50 times in order to reduce the force of contraction of canine papillary muscles by 50% (Yoneyama et al., 1990). The Ca^{2+} channel antagonistic effects on smooth muscle were not reversed by drug washout (Nishimura et al., 1990) and very long-lasting hypotensive effects were observed without changes in heart rate or AV conduction (Kageyama et al., 1991). This attractive spectrum of effects has stimulated various studies of semotiadil's Ca^{2+} channel antagonistic activities in both in vivo and in vitro experiments but studies on L-type Ca^{2+} channels have only been performed in smooth muscle cells of the vena portae (Teramoto, 1993). It was the aim of the present study to analyze the effects of semotiadil on cardiac L-type Ca^{2+} channels and to compare them with those of the well-known Ca^{2+} channel antagonists nifedipine, amlodipine and diltiazem. Measurements of the recovery of I_{Ca}

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from inactivation and of the potential dependence of drug influence proved semotiadil to be a use-dependent Ca^{2+} channel antagonist of the verapamil type. In its potency in I_{Ca} inhibition in guinea-pig ventricular cells semotiadil was comparable to diltiazem.

2. Materials and methods

2.1. Preparation of cardiomyocytes

Single cardiomyocytes from the ventricles (left and right) of adult guinea pigs were prepared as described by Piper et al. (1982). The coronary system of the heart was perfused in a Langendorff apparatus with a solution containing 100 IU/ml collagenase (Sigma, St. Louis, MO, USA). The isolated cells were kept in medium 199 (Biochrom, Berlin, Germany) supplemented with 5% calf serum (Gibco, Paisley, UK) and 100 IU/ml each of penicillin and streptomycin (Grünenthal, Stolberg, Germany). Experiments were performed on the day of preparation or on the following day.

2.2. Electrophysiological recordings

Voltage-clamp experiments (patch electrodes, whole-cell configuration; Hamill et al., 1981) were performed at 37°C in a Cs^+-Tyrode solution (to block K^+ currents) with the following composition (in mM): NaCl 137, CsCl 5.4, $NaHCO_3$ 2.2, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $CaCl_2$ 1.8, HEPES/Na^+ 10, D-(+)-glucose 5.6, pH 7.4. The patch pipette solution contained (in mM): CsCl 110, EGTA 11, $MgCl_2$ 2, $CaCl_2$ 1, HEPES/Na^+ 10, ATP/Na^+ 4.3, pH 7.4. Whole-cell recordings were made using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, USA). Voltage-clamp pulses were generated via an IBM-compatible computer connected to a D/A and A/D converter (Digidata 1200, Axon Instruments). Data aquisition and analyses were performed using pCLAMP software (Axon Instruments). Current recordings were started 5 min after breaking the patch to allow equilibration of the pipette solution with the cytosol.

2.3. General experimental protocol

The experiments were performed in an experimental bathing chamber (volume 1 ml) mounted on the stage of an inverted microscope (Axiovert 10, Zeiss, Oberkochen, Germany). The cells were superfused with warm (37°C) extracellular solution at the rate of 3 ml/min. The solution could be exchanged for an identical solution containing the substance under study without any significant alteration either in the flow rate or in the temperature of the superfusing fluid. A complete exchange of the bath solution was achieved within 1 min.

2.4. Drugs used in this study

Semotiadil (Santen Pharmaceutical, Japan) dissolved in dimethylsulfoxide, diltiazem (Goedecke, Germany), amlodipine (Pfizer, Austria), and nifedipine (Sigma, Germany) dissolved in physiologic saline were prepared before each experiment. Appropriate dilutions were made freshly for each experiment. The experiments with nifedipine were performed under yellow light.

2.5. Statistical analysis

For statistical analysis SPSS software (SPSS, Chicago, IL, USA) was used. Comparisons among groups were performed by analysis of variance (ANOVA) and least-significant difference contrasts. Control and drug data for individual groups were compared by Student's *t*-test. A probability of 5% was taken as indicating statistical significance.

3. Results

The inhibitory influence of 10 μM semotiadil on $I_{Ca,L}$ of a guinea-pig myocyte is shown in Fig. 1. The upper part of the figure gives the voltage protocol (stimulation fre-

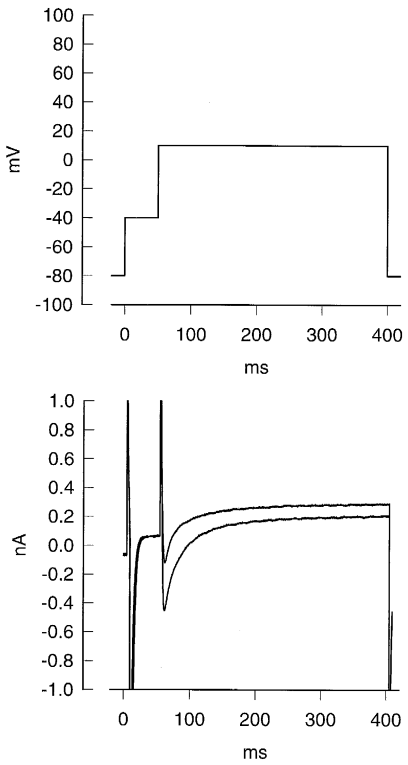

Fig. 1. Inhibitory influence of 10 μM semotiadil on $I_{Ca,L}$ of a guinea-pig ventricular cell. The upper part shows the voltage protocol, and the lower part gives original current tracings. I_{Na} was inactivated by a prepulse to −40 mV. The current recordings show I_{Ca} during control and 15 min after drug application. Stimulation frequency was 30/min.

quency was 30/min). In the lower part of the original current tracings are shown. A prepulse from a holding potential of −80 mV for 50 ms to −40 mV elicits I_{Na}, which can be seen as the downward deflection in both current tracings in the lower part. During the second voltage-clamp step the potential was changed from −40 mV to +10 mV for 350 ms. Under control conditions, this voltage step elicited I_{Ca} with an amplitude of 0.65 nA (difference between peak current and current at the end of the voltage pulse). The upper current tracing was obtained after superfusing the cell for 15 min with a solution containing 10 μM semotiadil. The amplitude of I_{Ca} was reduced by the drug in this particular experiment to 0.40 nA. The outward current at the end of the pulse to +10 mV (likely a Cs^+ current) was increased after inhibition of I_{Ca}. This indicates that I_{Ca} of the control is underestimated in this experiment by about 10%.

In Fig. 2 this inhibitory influence of semotiadil is shown in a more quantitative way in a concentration-response relationship and compared with the effects of diltiazem, nifedipine and amlodipine. The voltage-clamp protocol and stimulation frequency used were the same as in the experiment shown in Fig. 1. Semotiadil in a concentration of 1 μM produced an inhibition of 12.4 ± 9.7% and in a concentration of 10 μM an inhibition of 25 ± 11.0%. The IC_{50} for amlodipine and nifedipine was between 0.1 and 1 μM; for diltiazem it was between 10 and 100 μM.

In the experiment shown in Fig. 3 the recovery from inactivation of I_{Ca} was estimated under control conditions and under the influence of 0.1 μM semotiadil, 1 μM diltiazem, 0.01 μM nifedipine and 0.1 μM amlodipine. These low concentrations were chosen because I_{Ca} was elicited in these experiments from a holding potential of

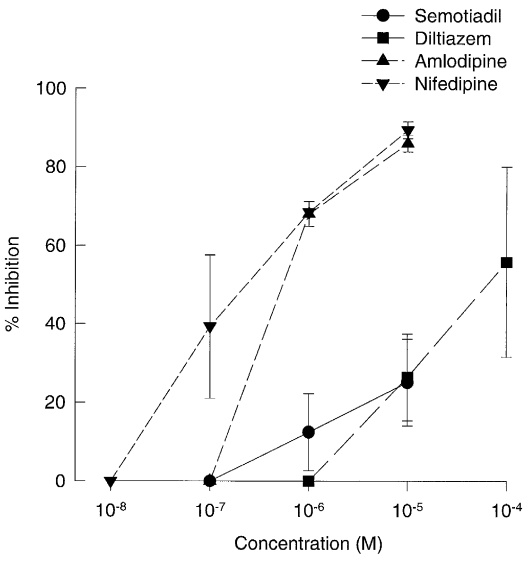

Fig. 2. Concentration-response relationship of the effects of semotiadil, diltiazem, amlodipine and nifedipine. IC_{50} of nifedipine and amlodipine was between 0.1 and 1 μM and that of semotiadil and diltiazem was between 10 and 100 μM. Number of experiments for each concentration: semotiadil and diltiazem 4, nifedipine and amlodipine 3.

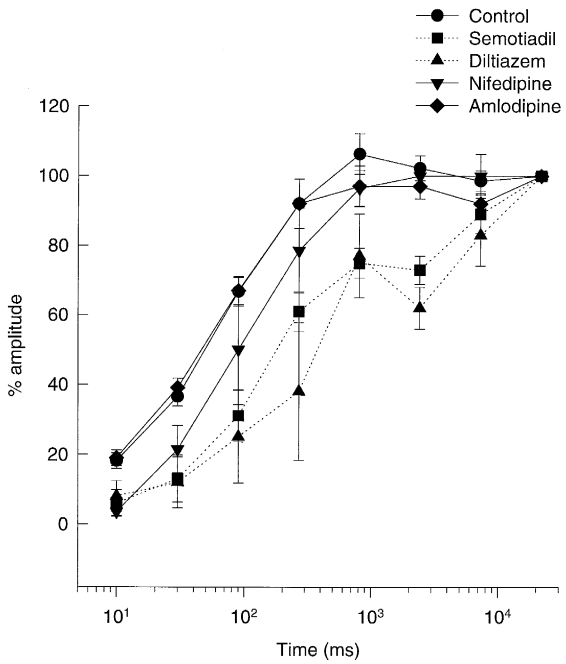

Fig. 3. Recovery of $I_{Ca,L}$ from inactivation under control conditions and under the influence of 0.1, 1, 0.01 and 0.1 μM semotiadil, diltiazem, nifedipine and amlodipine, respectively. In a double-pulse protocol the time between the two pulses was 10, 30, 90, 270, 810, 2430, 7290 and 21 870 ms. The curves indicate a distinct difference between the recovery under control as well as nifedipine and amlodipine conditions and under semotiadil and diltiazem conditions. The difference between the two groups was statistically significant at interpulse intervals of 270, 810 and 2430 ms. Number of experiments: control and semotiadil 5; diltiazem, nifedipine and amlodipine 3.

−40 mV. At this holding potential the effects of all four substances were more pronounced (cf., Table 1). The experimental procedure was similar to that used by Tseng (1988). I_{Ca} was elicited by pulses of 400 ms duration to +10 mV without a prepulse (frequency 3/min). In each experiment the recovery was first measured under control conditions. A double-pulse protocol of two successive voltage-clamp pulses was used, in which the time between the first and the second pulse was increased in eight steps from 10 to 30, 90, 270, 810, 2430, 7290 and 21 870 ms, respectively. After this control sequence the substance was

Table 1
Influence of the holding potential on the inhibitory effects of semotiadil, diltiazem, nifedipine and amlodipine on $I_{Ca,L}$

HP SF	−80 mV, 3/min	−80 mV, 30/min	−40 mV, 3/min
Semotiadil 0.1 μM	0	0	32.7 ± 11.2
Diltiazem 1 μM	0	0	74.7 ± 12.7
Nifedipine 0.01 μM	0	0	22.7 ± 0.12
Amlodipine 0.1 μM	0	0	17.0 ± 0.1

Data are given as % inhibition and as means ± S.E.M. The substances were applied in concentrations subthreshold for an inhibitory effect at a holding potential and pulse rate of −80 mV and 30/min, respectively. Number of experiments: semotiadil 5; diltiazem, nifedipine and amlodipine 3. HP, holding potential; SF, stimulation frequency.

added to the perfusion and the preparation was stimulated with single pulses with a frequency of 3/min for the following 15 min. After this, the recovery from inactivation was measured, using the double-pulse protocol a second time. For the estimation of current recovery, the current elicited by the second pulse of the last pair in the sequence after the longest interpulse interval of 21 870 ms was taken as 100%; the amplitude of the current during this pulse was in all experiments identical to the mean of the first and last three current recordings in the continuous recording period between the two double-pulse sequences. The current amplitudes after the shorter interpulse intervals are given relative to this value in Fig. 3. While there was no change in the time course of the recovery from inactivation by nifedipine and amlodipine, a prolongation of the recovery could be found after addition of semotiadil and diltiazem. Whereas in the control recovery was complete already after an interval of 810 ms (similar results were shown by Tseng, 1988), it was still incomplete after 2430 ms under the influence of semotiadil and diltiazem. The difference in the recovery at 270, 810 and 2430 ms between control, amlodipine and nifedipine on one hand and semotiadil and diltiazem on the other was statistically significant (ANOVA, least-significant difference contrasts). After 7290 ms there was no longer a significant difference between the two groups. These results indicate a distinct influence of heart rate on the effects of semotiadil and diltiazem and confirm the well-known lower dependence of nifedipine and amlodipine on this parameter.

The influence of the holding potential on the inhibitory effect of semotiadil in comparison with diltiazem, nifedipine and amlodipine was studied using holding potentials of -80 and -40 mV. The results are summarized in Table 1. The substance concentrations used were those shown not to cause inhibition at a holding potential of -80 mV and a stimulation frequency of 30/min (cf., Fig. 2). At -80 mV and 3/min also none of the substances caused inhibition. At -40 mV nifedipine and amlodipine in concentrations of 0.01 and 0.1 μM inhibited I_{Ca} by $22.7 \pm 0.12\%$ and $17.0 \pm 0.1\%$, respectively. The stronger inhibition of $32.7 \pm 11.2\%$ and $74.7 \pm 12.7\%$ produced by 0.1 μM of semotiadil and 1 μM of diltiazem may indicate a stronger dependence of the effects of these substances on the holding potential and likely a stronger effect in depolarized tissue. Statistical evaluation confirmed a significant difference between the efficacy of all four compounds at -80 and -40 mV (*t*-test), but the degree of current inhibition was not significantly different for the chosen concentrations of the four compounds (ANOVA, least-significant difference contrast).

4. Discussion

Semotiadil is a vasoselective Ca^{2+} channel antagonist. The present studies show that in guinea-pig ventricular cardiomyocytes, semotiadil inhibited L-type Ca^{2+} channels. The voltage-clamp experiments indicated that semotiadil is as potent as diltiazem in inhibiting peak Ca^{2+} currents but less potent than nifedipine and amlodipine. The different concentration-response relationships obtained confirm earlier findings for dihydropyridines and benzothiazepines (Koidl et al., 1988) and show that semotiadil's potency to inhibit I_{Ca} in ventricular myocytes is similar to that of diltiazem.

The use dependence of the I_{Ca} block, characterized by the dependence on the stimulation rate, pulse duration and holding potential, is significantly different within the group of Ca^{2+} channel antagonists (cf., McDonald et al., 1994). So, rapid pulsing increases the effect of phenylalkylamines and benzothiazepines more than that of dihydropyridines (Lee and Tsien, 1983; Uehara and Hume, 1985). Our study confirms these findings indirectly, by showing a slowing of the recovery from inactivation with semotiadil and diltiazem but not with nifedipine and amlodipine. These findings also confirm earlier data on slow-response action potentials in guinea-pig papillary muscles showing a rate-dependent $\dot{V}_{max}$ depression (Miyawaki et al., 1991). In addition, semotiadil's inhibitory effect was more pronounced when the membrane resting potential was clamped to -40 as compared to -80 mV. This marked potential dependence is common to all groups of Ca^{2+} channel antagonists (Sanguinetti and Kass, 1984; Kass and Arena, 1989). The marked rate dependence of the channel blocking activity of semotiadil indicates a use-dependent mode of action in accordance to the modulated receptor hypothesis (Hondeghem and Katzung, 1984). Similar findings were obtained for canine AV nodes showing frequency-dependent prolongation of the functional refractory period by semotiadil (Kageyama et al., 1991).

These data are in accordance with further findings (not shown) demonstrating no obvious modification of activation and inactivation processes. Hence, semotiadil seems primarily to modulate channel conductance but not the gating characteristics of the channels.

Kass and Arena (1989) reported that amlodipine as an ionized dihydropyridine derivative produces a frequency-dependent inhibition whereas the neutral form produces a tonic inhibition of the cardiac Ca^{2+} channel. Amlodipine did not inhibit the Ca^{2+} channel when it was applied to the cytosol. In a similar fashion, semotiadil produced no inhibition of I_{Ca} when applied to the cytosol (Teramoto, 1993). There results and our findings lead to the suggestion that semotiadil acts on the Ca^{2+} channel from outside, probably via strong dissolution in the membrane. Teramoto (1993) has discussed that semotiadil in the neutral form may act on the Ca^{2+} channel in the resting state through a hydrophobic site of the channel and that the ionized drug may act on the Ca^{2+} channel mainly in the inactivated state (at pH 7.3).

In conclusion, semotiadil is a structurally different new Ca^{2+} channel antagonist with a moderate efficacy to block

transmembrane inward Ca^{2+} currents in the myocardium. The strong use dependence of the inhibitory action of semotiadil indicates a verapamil-type of action. Our and other findings (Teramoto, 1993) indicate that semotiadil binds predominantly to the inactivated channel state and, thus, inhibits the Ca^{2+} channel in a strong rate- and potential-dependent mode, probably by slowing the transition rate from the inactivated to the rested state. This inhibitory effect in cardiomyocytes is known to be accompanied by strong vasolidating properties. A concentration of semotiadil that caused a borderline AV conduction prolongation (by 15%) already doubled coronary blood flow (Yoneyama et al., 1990). Our data provide direct evidence for the antagonistic potency of semotiadil which is in accordance with many earlier studies (Miyawaki et al., 1990, 1991; Nishimura et al., 1990; Yoneyama et al., 1990, Kageyama et al., 1991; Takada et al., 1991; Nakayama et al., 1992, 1994).

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