Effect of S16257 on L-type Calcium Current in Frog and Rat Ventricular Myocytes

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Myocardial ischaemia is often accompanied by increased heart rate (HR). Regular pharmacological agents used to reduce HR have an undesirable negative inotropic side-effect. For this reason the inhibitors of pacemaker current (I_f) such as S16257 recently have been developed and are supposed to reduce HR selectively. We used whole-cell patch-clamp for studying the effect of S16257 on L-type Ca^{2+} current (I_{Ca}), which determines myocardial contraction force, in frog and rat ventricular myocytes. **In frog** ventricular myocytes 0.001–0.01 mM of S16257 slightly stimulated ($p > 0.05$) I_{Ca}, however, higher concentrations exerted a concentration-dependent inhibition of I_{Ca}. Half concentration for inhibition (IC_{50}) was 0.41 mM. The peak of current–voltage dependence curve was shifted from 0.1 ± 0.8 mV in control to –16.1 ± 1.6 mV in the presence of 1 mM of S16257. The inactivation of I_{Ca} by 50% was obtained at –36.4 ± 1.3 mV in control and at –42.2 ± 0.9 mV in the presence of 1 mM of S16257. **In rat** ventricular myocytes 0.01–10 mM of S16257 exerted a concentration-dependent inhibition of I_{Ca}. IC_{50} was 0.52 mM. The peak of current–voltage dependence curve was shifted from 1.6 ± 1.2 mV in control to –5.3 ± 0.9 mV in the presence of 1 mM of the compound. The inactivation of I_{Ca} by 50% was obtained at –29.9 ± ± 1.0 mV in control and at –31.2 ± 1.0 mV in the presence of 1 mM of S16257. The effect of S16257 on I_{Ca} in both species was completely reversible. The results show that in frog and rat ventricular myocytes S16257 at concentrations higher than 0.01 mM exert a concentration-dependent dihydropyridine-antagonists-like inhibitory effect on I_{Ca}. This inhibitory effect is dependent on the membrane potential only in frog ventricular myocytes.

Key words: S16257, patch-clamp, L-type calcium current, frog and rat ventricular myocytes

INTRODUCTION

Ischaemic heart diseases are often accompanied by increased heart rate (HR). Sinus tachycardia increases cardiac output and in this way helps to maintain homeostasis, however, it also strongly increases myocardial oxygen demand. This imbalance between oxygen supply and demand may lead to irreversible myocardial injury. For this reason beta-adrenoceptor antagonists and calcium channel blockers are often used in patients with angina pectoris, ischaemic heart diseases and congestive heart failure. However, these agents not only reduce the heart rate, but also exert negative inotropic and hypotensive effects. Development of new agents selectively reducing HR without undesirable side effects offers new possibilities in treatment of ischaemic heart diseases. These drugs act by inhibiting the hyperpolarization-activated pacemaker current (I_f) in the sinoatrial node (1).

The recently developed inhibitor of I_f, S16257, has been shown to have bradycardic action with no signs of negative inotropic action in conscious rats (2). Just an initial depressor effect was observed, which could be associated with reductions in renal, mesenteric and hindquarters flow. In conscious dogs S16257 has been shown not to display any negative inotropic effect or any vasoconstrictor effect at the level of large and small coronary arteries, either at rest or at exercise (10). In rabbit and guinea-pig cardiac preparations S16257 inhibited the diastolic

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rate of depolarization (3), but produced some increase in repolarization time, what could be a cause of polymorphic ventricular tachyarrhythmias (4). Again, in guinea-pig isolated cardiac preparations S16257 has been shown, additionally to its bradycardic action, to be a sodium channel blocker, what can lead to the suppression of cardiac arrhythmias in an ischaemic myocardium (3).

In the present work we examined the concentration-dependent effects of S16257 (7,8-dimethoxy 3-{3-{[(1S)-(4,5-dimethoxybenzocyclobutan-1-yl) methyl] methylamino}propyl} 1,3,4,5-tetrahydro-2H-benzazepin 2-one) on the amplitude, current–voltage relationship and inactivation of L-type calcium current (I_{Ca}) in frog and rat single ventricular myocytes. Since the major determinant of the myocardial contraction force is I_{Ca}, we expect that these pharmacological characteristics of the compound would favour its clinical application.

MATERIALS AND METHODS

Frog ventricular myocytes were enzymatically dispersed from frog (*Rana esculenta*) heart by a combination of collagenase (Yakult, Japan) and trypsin (type XIII, Sigma, USA) as described (5). Frogs were decapitated and double-pitched. The isolated cells were stored in storage Ringer solution and kept at 4 °C until use (2–48 h after dissociation). In some isolations, amino acids were omitted from the dissociation and storage solutions, with no change in the results.

Rat ventricular myocytes were obtained by retrograde perfusion from hearts of male Wistar rats (180–220 g) as previously described (6). The myocytes were maintained at 37 °C until use.

Solutions. For electrophysiology, the control external solution contained (in mM): NaCl 107; HEPES 10; CsCl 20; $NaHCO_3$ 4; NaH_2PO_4 0.8; $MgCl_2$ 1.8; $CaCl_2$ 1.8; D-glucose 5; sodium pyruvate 5; tetrodotoxin 3×10^{-4} (for frog) or 6×10^{-3} (for rat); pH 7.4 adjusted with NaOH. Patch electrodes (0.6–1.5 Mohms) were filled with control internal solution which contained (mM): CsCl 119.8; EGTA (acid form) 5; $MgCl_2$ 4; creatine phosphate disodium salt 5; Na_2ATP 3.1; Na_2GTP 0.42; $CaCl_2$ 0.062 (pCa 8.5); HEPES 10; pH 7.1 (frog) or 7.3 (rat) adjusted with CsOH. Collagenase type A for rat cardiac myocyte dissociation and fetal calf serum were from Boehringer Mannheim (Germany). DMEM was obtained from Gibco-BRL. Tetrodotoxin (TTX) was from Latoxan (Rosans, France). S16257 was from Servier (Neuilly-sur-Seine, France). All other drugs were from Sigma Chemical Co. (St. Louis, MO). All drugs tested in patch-clamp experiments were solubilized in experimental solutions just before application onto the cell studied, *i.e.*, only fresh solutions were tested.

Electrophysiological experiments. The whole cell configuration of the patch-clamp technique was used to record the high-threshold I_{Ca} on Ca^{2+}-tolerant frog and rat ventricular myocytes. In the routine protocols the cells were depolarized every 8 s from a holding potential of –80 to 0 mV for 200 or 400 ms. In rat cardiomyocytes, the test pulse to 0 mV was preceded by a short pre-pulse (50 ms) to –50 mV. The prepulse and/or the application of TTX were used to eliminate fast sodium currents. K^+ currents were blocked by replacing all intracellular and extracellular K^+ ions with Cs^+. For determination of current–voltage relationships for I_{Ca} and I_{Ca} inactivation curve a double pulse voltage-clamp protocol was used. Every 4 s the membrane potential of the cell, which was normally maintained at its holding value of –80 mV, experienced the following sequence of events: different potential values ranging from –100 to +100 mV for 200 ms, –80 mV for 3 ms and 0 mV for 200 ms (see inset in Fig. 2B). Voltage clamp protocols were generated by a challenger/09–VM programmable function generator (Kinetic Software, Atlanta, GA). The cells were voltage-clamped using a patch-clamp amplifier (model RK-400; Bio-Logic, Claix, France). Currents were sampled at a frequency of 10 kHz using a 16-bit analogue-to-digital converter (PCL816, Advantech France, Levallois Perret, France).

Control and drug-containing solutions with 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM of S16257 were applied to the exterior of the cell by placing the cell at the opening of a 0.3 mm inner diameter capillary tubings flowing at a rate of about 50 µl/min. Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC100, Bio-Logic, Claix, France). All experiments were carried out at room temperature.

Data analysis. The maximal amplitude of whole-cell I_{Ca} was measured as previously described (5, 7). The currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was done to determine, for each membrane depolarization, the peak and steady-state current values. Cumulative dose–response curves were obtained by testing 4 (for rat) or 5 (for frog) successively increasing concentrations of S16257 on I_{Ca}. For each concentration of S16257, a percentage variation in I_{Ca} amplitude with respect to its control level was calculated: (% variation of I_{Ca}) = 100[(I_{Ca} with S16257) – (control I_{Ca})]/(control I_{Ca}). For each I_{Ca} inhibiting concentration of S16257, a percentage inhibition of I_{Ca} amplitude with respect to its maximal level was calculated: (% of maximal I_{Ca}) = 100(I_{Ca} with

S16257)/(maximal I_{Ca}). Averaged values of several experiments are represented as mean ± S.E.M., fit to the Michaelis equation and the half concentration (IC_{50}) of S16257 for inhibition of I_{Ca} derived. Differences between means were tested for statistical significance by Student's t test.

RESULTS

Effect of S16257 on I_{Ca} in frog ventricular myocytes.

A typical experiment in Fig. 1 shows the effect of S16257 on I_{Ca} in frog ventricular myocytes. During the periods indicated by horizontal lines, the cell was successively exposed to five increasing concentrations of S16257 (0.001, 0.01, 0.1, 1 and 10 mM). Each symbol represents the maximal peak amplitude of I_{Ca} obtained by depolarising the cell every 8 s to 0 mV over a period of 200 ms from a holding potential of –80 mV. The current traces shown in the upper part of Fig. 1 were recorded at the times indicated by corresponding letters on the main graph. Initially the I_{Ca} was measured in control conditions and its amplitude was about 125 pA. The application of the first concentration of S16257 (0.001 mM) increased the I_{Ca} by about 14.1%. The second concentration of the compound (0.01 mM) did not exert any additional effect, however, the application of the next three increasing concentrations of S16257 evoked a dose-dependent inhibition of I_{Ca}, and at the presence of 10 mM the I_{Ca} was completely blocked. After washout of the compound the I_{Ca} amplitude returned close to its initial control value. This shows that the effect of S16257 is completely reversible. In other words, short application of 10 mM S16257 has no apparent toxic effect on the frog ventricular myocytes. The current traces of Fig. 1 show that the effects of S16257 are reached essentially by changes in I_{Ca} amplitude with no apparent changes in the activation and inactivation of the I_{Ca}, suggesting that the compound does not change the opening-closure characteristics of calcium channels. To analyse this more precisely, we applied to the myocytes membrane potentials from –100 mV to +100 mV to obtain the curves of current–voltage dependence (Fig. 2A). In the same protocol the second depolarization to 0 mV (insert in Fig. 2B) allowed to obtain also the inactivation curve of I_{Ca} as a function of the prepulse potential (Fig. 2B). Average curves in Fig. 2A are expressed in current density and calculated from 5 experiments in control and in the presence of 1 mM S16257. I_{Ca} begins to activate at about a –40 mV potential and reaches its maximal value at about 0 mV. Then it progressively decreases when the membrane potential approaches to the Ca^{2+} equilibrium potential. Actually I_{Ca} was maximal

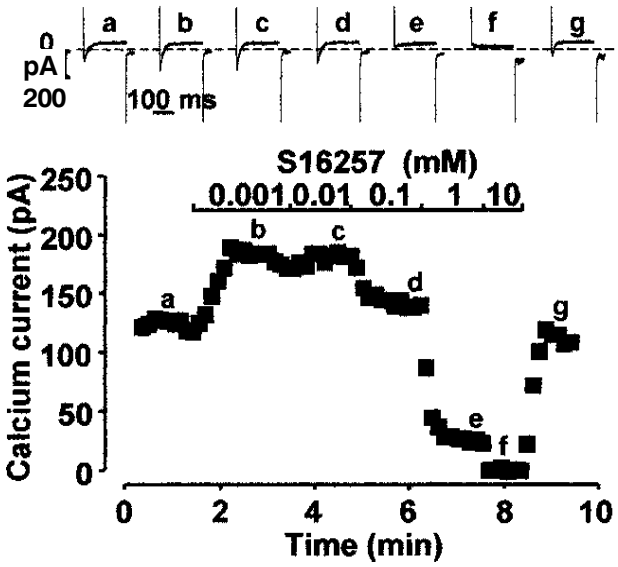

Fig. 1. Effect of S16257 on I_{Ca} in frog ventricular cell. A frog ventricular myocyte was initially superfused with control external solution and internally dialysed with control internal solution. Each symbol represents the maximal peak amplitude of I_{Ca}. During the periods indicated by the horizontal line, the cell was successively exposed to five increasing concentrations of S16257 (0.001, 0.01, 0.1, 1 and 10 mM). The current traces shown in the upper part of the figure were recorded at the times indicated by the corresponding letters on the main graph

at 0.1 ± 0.8 mV in control and at –16.1 ± 1.6 mV in the presence of 1 mM S16257 ($n = 5$; $p < < 0.0005$). The peak of the current–voltage dependence curve was shifted by 16 mV to the negative potentials.

Inactivation curves in Fig. 2B also characterize the I_{Ca} (5). When the membrane potential increases from –100 mV to 0 mV, the degree of inactivation increases up to 100% of inactivation or 0% of the availability of calcium channels. Successively increasing the membrane potential from 0 mV to +100 mV, the inactivation becomes progressively less. This shows that the inactivation depends at the same time on the membrane potential and on the influx on Ca^{2+} ions into the cell (7). For this reason the current is less inactivated when the influx of Ca^{2+} ions is reduced. Inactivation of I_{Ca} by 50% was obtained at –36.4 ± 1.3 mV in control conditions and at –42.2 ± 0.9 mV in the presence of 1 mM S16257 ($n = 5$; $p < 0.01$). In this way a shift of the inactivation curve by about 6 mV to the negative potentials was observed, and it was less than that of current–voltage dependence.

The variations of the I_{Ca} amplitude in the presence of S16257 are summarized in Fig. 3 and Table 1. Figure 3A shows mean and standard error values of the calcium current density. The effects of 5

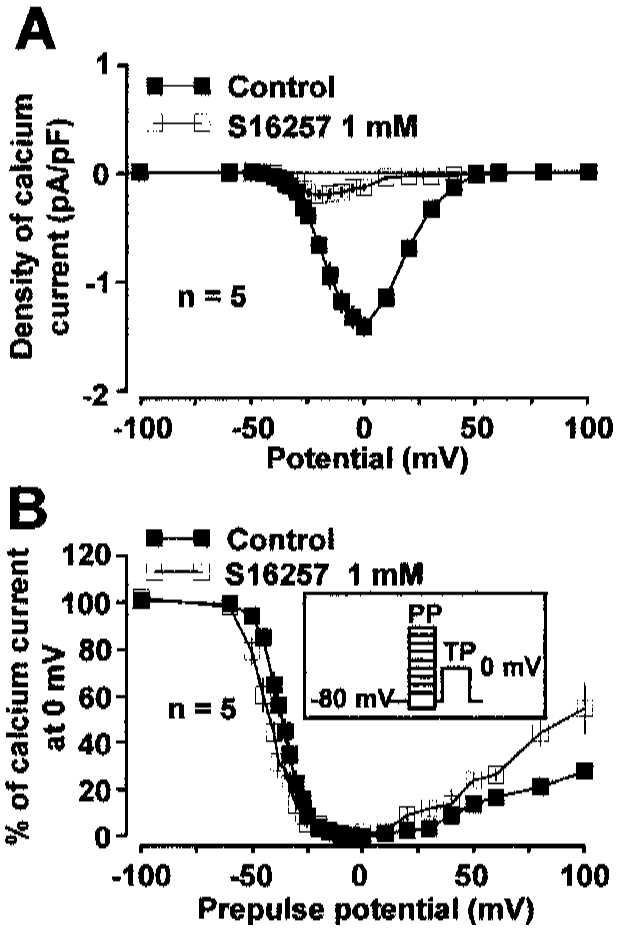

Fig. 2. Voltage dependence of the inhibitory effect of S16257 on I_{Ca} in frog ventricular myocytes. Current–voltage curves (A) and inactivation curves (B) of I_{Ca} in control conditions and in the presence of 1 mM S16257. The points show the mean and the vertical lines S.E.M. of the number of cells indicated. The current–voltage relationships of I_{Ca} were measured during 200 msec depolarizations to various potentials. Inactivation curves of I_{Ca} were obtained using a double-step protocol consisting of a 200 ms prepulse (PP) to various potentials followed by 200 ms test potential (TP) to 0 mV as indicated in the inset. I_{Ca} measured during a 200 ms test pulse at 0 mV is plotted as a function of the prepulse potential and expressed as a percentage of I_{Ca} at 0 mV in the absence of prepulse. Maximal I_{Ca} was measured at 0.1 ± 0.8 mV in control and at –16 ± 1.6 mV in the presence of 1 mM S16257 ($p < 0.0005$). The inactivation of I_{Ca} by 50% was obtained at –36.4 ± 1.3 mV in control and at –42.2 ± 0.9 mV in the presence of 1 mM S16257 ($p < 0.01$)

concentrations of S16257 in 10 experiments are compared to control current densities. 0.001–0.01 mM of S16257 evoked a small increase of I_{Ca}, however, with application of higher concentrations of the compound a progressive inhibition of I_{Ca} was observed. Figure 3B summarizes the inhibitory effects of S16257 in 10 experiments. The symbols show the mean experimental data, and a continuous line was

Table 1. **Summary of the effects of S16257 on calcium current density in frog ventricular myocytes**

[S16257], mM	Density of I_{Ca} (pA/pF) (mean ± S.E.M.)	n
0	1.63 ± 0.04	10
0.001	1.66 ± 0.15	10
0.01	1.86 ± 0.15	10
0.1	1.59 ± 0.09	10
1	0.38 ± 0.04	10
10	0.0 ± 0.0	10

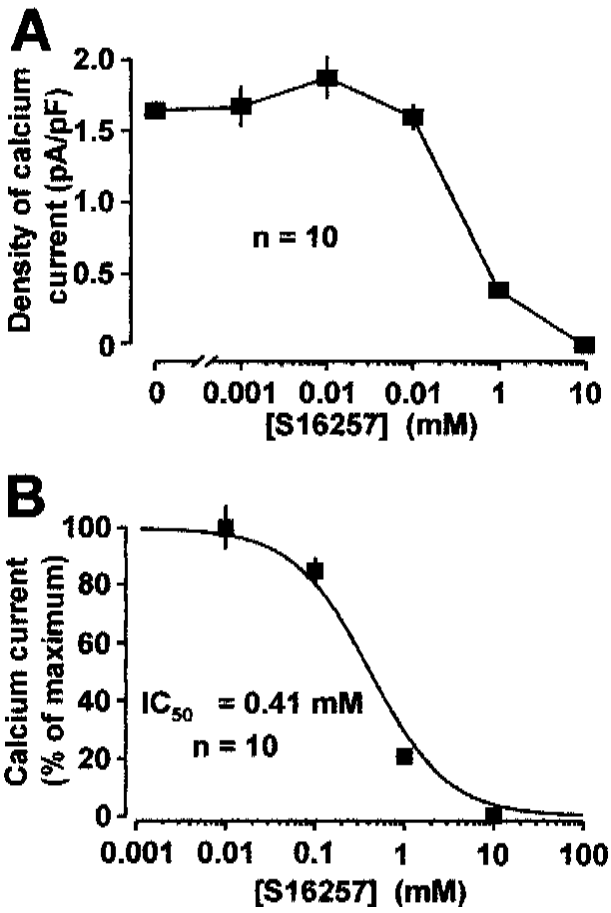

Fig. 3. Concentration–response effects of S16257 (A) and concentration–response curve for the inhibiting effect of S16257 (B) on I_{Ca} in frog ventricular myocytes. The points show the mean and the vertical lines S.E.M. of the number of cells indicated. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation. The concentration of S16257 (IC_{50}) required for half-maximal inhibition of I_{Ca}, derived from this analysis, was 0.41 mM

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Effect of S16257 on I_{Ca} in rat ventricular myocytes.

A typical experiment in Fig. 4 shows the effect of S16257 on I_{Ca} in rat ventricular myocytes. During the periods indicated by horizontal lines, the cell was successively exposed to four increasing concentrations of S16257 (0.01, 0.1, 1 and 10 mM). The current traces shown in the upper part of Fig. 4 were recorded at the times indicated by corresponding letters on the main graph. Initially the I_{Ca} was measured in control conditions and its amplitude was about 500 pA. The application of the first concentration of S16257 (0.01 mM) reduced the I_{Ca} by about 10%. Higher concentrations progressively inhibited I_{Ca}, and at 10 mM of S16257 the calcium current was not measurable any more. After the washout of the compound the I_{Ca} amplitude returned close to its initial control value, and this shows that the effect of 10 mM S16257 is completely reversible, as well as in frog ventricular myocytes.

Figure 5 shows the average curves of current–voltage dependence (Fig. 5A) and inactivation (Fig. 5B) of I_{Ca} obtained from 4 experiments in control and in the presence of 1 mM S16257. Both curves have the same shape as obtained from the frog cells. The application of 1 mM S16257 strongly inhibits the amplitude of I_{Ca} and slightly shifts the peak of current–voltage dependence curve to the negative potentials. However, this shift by about 7 mV is less than in frog myocytes. I_{Ca} was maximal at 1.6 ± 1.2 mV in control and at –5.3 ± 0.9 mV in the presence of 1 mM S16257 (n = 4; p > 0.05). The same shape of the I_{Ca} inactivation curve was

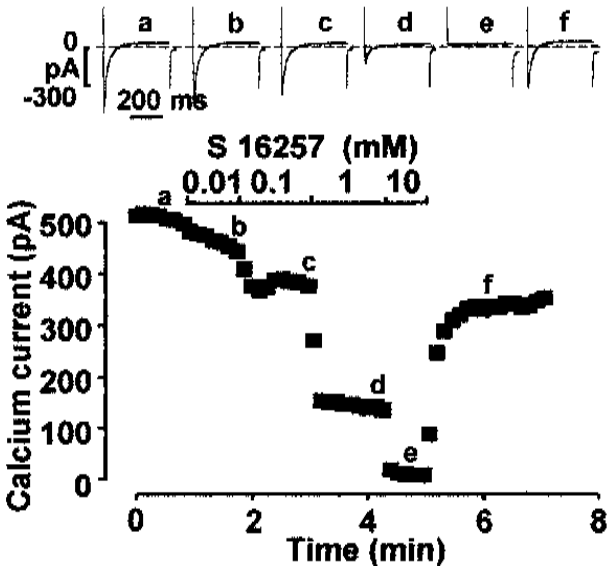

Fig. 4. Effect of S16257 on I_{Ca} in rat ventricular cell. A rat ventricular myocyte was initially superfused with control external solution and internally dialysed with control internal solution. Each symbol represents the maximal peak amplitude of I_{Ca}. During the periods indicated by the horizontal line, the cell was successively exposed to four increasing concentrations of S16257 (0.01, 0.1, 1 and 10 mM). The current traces shown in the upper part of the figure were recorded at the times indicated by the corresponding letters on the main graph

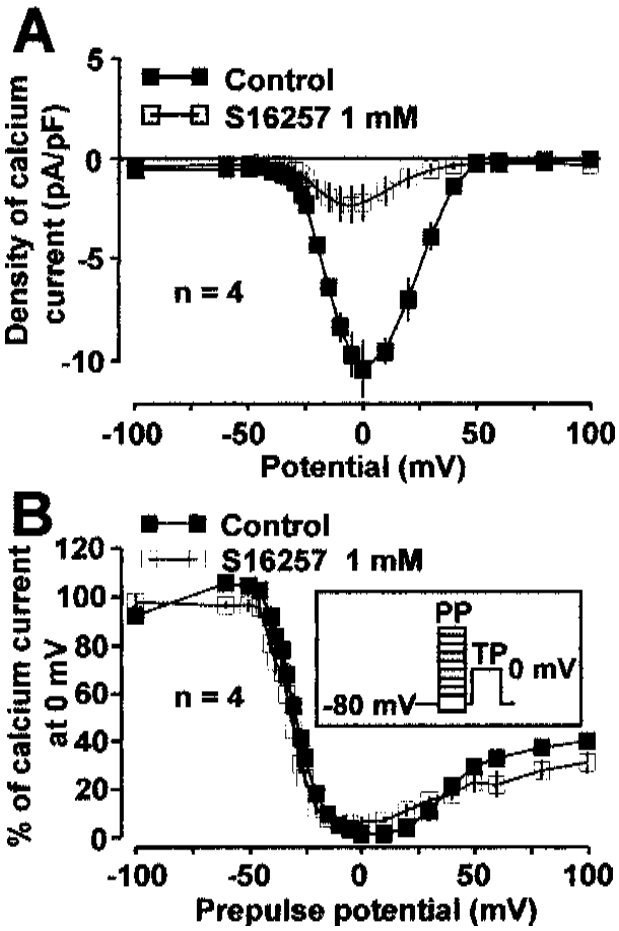

Fig. 5. Voltage dependence of the inhibitory effect of S16257 on I_{Ca} in rat ventricular myocytes. Current–voltage curves (A) and inactivation curves (B) of I_{Ca} in control conditions and in the presence of 1 mM S16257. The points show the mean and the vertical lines S.E.M. of the number of cells indicated. The current–voltage relationships of I_{Ca} were measured during 200 ms depolarizations to various potentials. Inactivation curves of I_{Ca} were obtained using a double-step protocol consisting of a 200 ms prepulse (PP) to various potentials followed by a 200 ms test potential (TP) to 0 mV as indicated in the inset. I_{Ca} measured during a 200 ms test pulse at 0 mV is plotted as a function of the prepulse potential and expressed as a percentage of I_{Ca} at 0 mV in the absence of prepulse. Maximal I_{Ca} was measured at 1.6 ± 1.2 mV in control and at –5.3 ± 0.9 mV in the presence of 1 mM S16257 (p > > 0.05). The inactivation of I_{Ca} by 50% was obtained at –29.9 ± 1.0 mV in control and at –31.2 ± 1.0 mV in the presence of 1 mM S16257 (p > 0.05)

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The variations of the I_{Ca} amplitude in the presence of S16257 are summarized in Fig. 6 and Table 2. Figure 6A shows the mean and standard error values of the calcium current density. The effects of 4 concentrations of S16257 in 13 experiments are compared to control current densities. 0.01–10 mM of S16257 evoked a progressive inhibition of I_{Ca}. Figure 6B summarizes the inhibitory effects of S16257 in 13 experiments. The symbols show the mean experimental data, and a continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation. The concentration of S16257 (IC_{50}) required for half maximal-inhibition of I_{Ca} derived from this analysis was 0.52 mM.

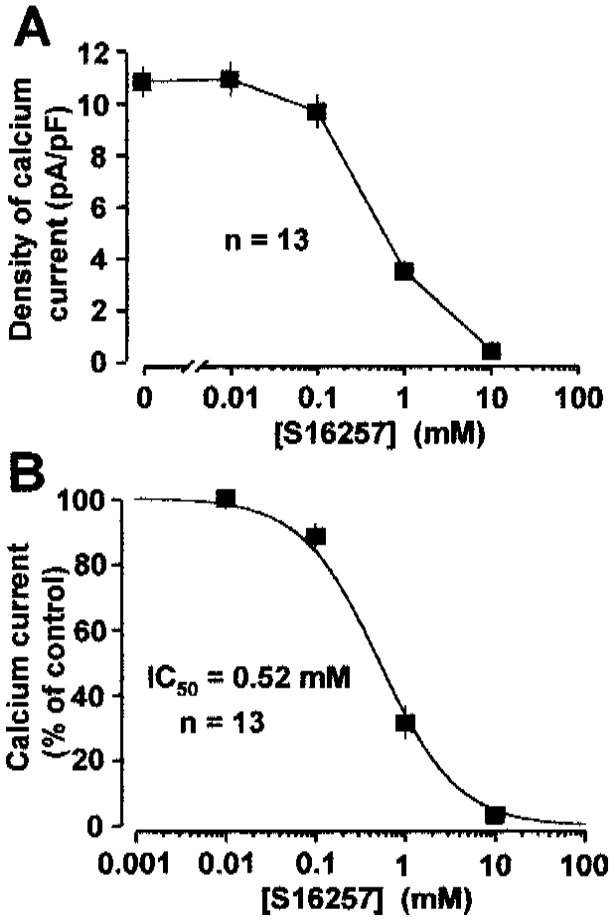

Fig. 6. Concentration–response effects of S16257 (A) and a concentration–response curve for the inhibiting effect of S16257 (B) on I_{Ca} in rat ventricular myocytes. The points show the mean and the vertical lines S.E.M. of the number of cells indicated. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation. The concentration of S16257 (IC_{50}) required for half-maximal inhibition of I_{Ca}, derived from this analysis, was 0.52 mM

Table 2. **Summary of the effects of S16257 on calcium current density in rat ventricular myocytes**

[S16257], mM	Density of I_{Ca} (pA/pF) (mean ± S.E.M.)	n
0	10.81 ± 1.14	13
0.01	10.91 ± 1.26	13
0.1	9.66 ± 1.17	13
1	3.52 ± 0.67	13
10	0.45 ± 0.15	13

DISCUSSION

This study has compared the effects of S16257, the inhibitor of pacemaker current, on L-type calcium current in frog and rat ventricular myocytes. In frog, 0.001–0.01 mM of S16257 had no inhibitory effect, but slightly (p > 0.05) stimulated I_{Ca}. However, in frog as well as in rat myocytes, all higher concentrations of the compound had pronounced concentration-dependent inhibitory effects, up to complete block of I_{Ca}. The half concentration for inhibition of the calcium current was 0.41 mM in the frog and 0.52 mM in the rat. The maximal effect was reached at 10 mM in both species. This corresponds to the results reported by Perez and co-workers (9), who found that S16257 inhibited the contraction force of guinea-pig isolated cardiac preparations at concentrations higher than 0.01 mM. No apparent toxic effect of the compound was observed, since 2–3 min exposures of the myocytes to this concentration of S16257 had no irreversible action.

The inhibitory effects of S16257 were dependent on the membrane potential, and this dependence was more remarkable in the frog than in the rat. A shift of the current–voltage dependence curve to the side of negative potentials was observed. In other words, S16257 inhibits I_{Ca} more effectively when the cell is depolarized, and this property of the compound corresponds to the inhibitory effects of dihydropyridine antagonists on I_{Ca}. Actually, such compounds as nifedipine or nitrendipine exert the inhibitory effects on I_{Ca} that are more pronounced at depolarized potentials than at hyperpolarized ones (8). This phenomenon possibly is due to a higher affinity of dihydropyridine antagonists to inactivated than to resting calcium channels. In the case of nifedipine, the depolarization of the membrane potential from –80 mV to –40 mV augments the affinity of the compound to calcium channels by two orders (8). In frog ventricular myocytes, S16257 at high concentrations also could exert its inhibitory

effect by the mechanism similar to that of dihydropyridine antagonists. Although the action of dihydropyridine antagonists on I_{Ca} has the same characteristics in amphibians and in mammalians, in this study we have observed that in the rat, differently than in the frog, the inhibitory effects of S16257 were not accompanied by a shift of the current voltage dependence curve. To verify whether this difference does exist among the species, it could be useful to test the compound on other mammalian or, best of all, on human cardiac myocytes.

In conclusion, it should be noted that S16257, at a concentration range of 0.001–0.01 mM, which is reported by other authors (2, 3, 10) to reduce effectively the heart sinus rate in rat, dog and guinea-pig, had no effect on the L-type calcium current in frog and rat ventricular myocytes. However, the higher concentrations of S16257 should be used with precaution because of its strong inhibitory effect on I_{Ca}, which directly regulates the myocardial contraction force.

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S16257 POVEIKIS L-TIPO KALCIO SROVEI VARLĖS IR ŽIURKĖS KARDIOMIOCITUOSE

S a n t r a u k a

Širdies išeminę ligą dažnai lydi padažnėjęs širdies ritmas. Siekiant jį sulėtinti, neslopinant širdies susitraukimo jėgos, gali būti panaudotos bradikardinės medžiagos – peismeikerinės srovės (I_f) blokatoriai. Tirtas I_f blokatoriaus S16257 poveikis L-tipo kalcio srovei (I_{Ca}) varlės ir žiurkės skilvelių miocituose. Varlės miocituose 0,001–0,01 mM S16257 nestipriai stimuliavo I_{Ca}, tačiau didesnės medikamento koncentracijos I_{Ca} slopino iki visiško blokavimo esant 10 mM koncentracijai. Pusinė blokavimo koncentracija (IC_{50}) sudarė 0,41 mM. 1 mM S16257 pastūmėjo srovės – potencialo priklausomybės kreivės piką nuo 0,1 ± 0,8 mV iki –16,1 ± ± 1,6 mV. I_{Ca} 50% inaktyvuota buvo esant –36,4 ± 1,3 mV (kontrolė) bei –42,2 ± 0,9 mV, paveikus 1 mM S16257. Žiurkės miocituose priklausomai nuo koncentracijos 0,01–10 mM S16257 slopino I_{Ca}. 10 mM medikamento srovę visiškai blokavo. IC_{50} nustatyta 0,52 mM. Žiurkės miocituose 1 mM S16257 pastūmėjo srovės – potencialo priklausomybės kreivės piką nuo 1,6 ± 1,2 mV iki –5,3 ± 0,9 mV. I_{Ca} 50% inaktyvuota buvo esant –29,9 ± 1,3 mV (kontrolė) bei –31,2 ± 1,0 mV, paveikus 1 mM S16257. Medikamento poveikis tiek varlės, tiek žiurkės miocituose buvo grįžtamojo pobūdžio. Rezultatai rodo, jog didesnės nei 0,01 S16257 koncentracijos varlės ir žiurkės miocituose slopina I_{Ca} panašiai kaip dihidropiridino antagonistai, tačiau šis poveikis priklauso nuo membranos potencialo tik varlės miocituose.

Raktažodžiai: S16257, peismeikerinės srovės blokatorius, L-tipo kalcio srovė, varlės ir žiurkės skilvelių miocitai