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Calmodulin antagonist W7 directly inhibits f-type current in rabbit sino-atrial cells

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Abstract

As reported for cyclic nucleotide-gated channels in sensory neurons, we investigated the action of Ca^{2+}–calmodulin and calmodulin antagonist (W7) on the apparent affinity of pacemaker (I_f) channels for cAMP.

In this study, we used the patch-clamp technique in inside–out macro-patch configuration in rabbit sino-atrial cells. Intracellular calmodulin perfusion had no effect on f-channel activity and did not change the cAMP-induced I_f activation shift. Nevertheless, W7 decreased maximal conductance and induced a voltage shift of the current activation curve towards negative potentials. W7 did not modify the positive shift caused by cAMP, and cAMP did not prevent the effects of W7.

Contrary to the cyclic nucleotide-gated channel, the f-channel is not directly modulated by Ca^{2+}–calmodulin. The data suggest that W7 alters the voltage-dependent properties of I_f independent of cAMP binding. This agent opens the pathway for a new family of bradycardic drugs.

Keywords: Calcium–calmodulin; Hyperpolarization-activated channel; cAMP; Sino-atrial

1. Introduction

The funny current, or pacemaker (I_f) current, was first described in cardiac pacemaker cells of the mammalian sino-atrial node as a current that slowly activates on hyperpolarization at voltages in the diastolic voltage range, and contributes to the generation of cardiac rhythmic activity and to its control by sympathetic and parasympathetic innervations (DiFrancesco et al., 1986). The f-current described in other cardiac regions and in a variety of neuronal cells (for reviews; Accili et al., 2002; Robinson and Siegelbaum, 2003), is a nonspecific cationic current carried by K^+ and Na^+ ions. In sino-atrial cells, f-channels are modulated by cAMP independently of phosphorylation, through a mechanism involving direct interaction of cAMP with the intracellular side of the channels (DiFrancesco and Tortora, 1991; Bois et al., 1997). A significant advancement in the study of molecular properties of pacemaker channels was achieved when a new family of channels was cloned, the HCN (hyperpolarization-activated, cyclic nucleotide gated) (Biel et al., 2002) channels. The HCN family is related to the cyclic nucleotide-gated channel and *eag* potassium channel family and belongs to the superfamily of voltage-gated cation channels. HCN channels are characterized by six membrane-spanning segments (S1–S6) including a voltage-sensing (S4) and pore region (between S5 and S6). In the C-terminal region they contain a consensus sequence for binding of cyclic nucleotides. In the heart, neurotransmitter-induced control of cardiac rhythm is mediated by I_f through its second-messenger cAMP, whose synthesis is stimulated and inhibited by β-adrenoceptor and muscarinic agonists, respectively. Cyclic nucleotide binding on the intracellular side of the channel results in a shift of the current activation curve to more positive voltages with no change in maximal conductance (DiFrancesco and Tortora, 1991). Direct activation by cyclic nucleotides is one of the properties that f-channels share with cyclic nucleotide-gated

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channels found in photoreceptors and olfactory receptor neurons (Kaupp, 1991). In the olfactory receptor, the apparent affinity of the cyclic nucleotide-gated channel for cAMP is significantly reduced in the presence of Ca^{2+}–calmodulin. This decrease in apparent affinity appears to involve an interaction between Ca^{2+}–calmodulin and the channel (Chen and Yau, 1994). Recently, Rigg et al. (2003) reported that the f-current recorded in guinea-pig sino-atrial node cells was sensitive to the calmodulin antagonist W7. This antagonist consistently shifted the voltage of half-activation to a more negative potential without significantly altering the slope of activation. Contrary to the L-type calcium current (Vinogradova et al., 2000), the calmodulin dependent kinase (CaMKII) antagonist (KN-93) had no effect on either the amplitude or voltage dependence of the pacemaker current. This indicates that modulation of the f-current by W7 would be independent of CaMKII.

To evaluate a possible direct action of both calmodulin and W7 on I_f, we have investigated the action of these two molecules on the intracellular side of f-channels in inside–out macro-patches.

2. Materials and methods

2.1. Sino-atrial cell isolation

The methods employed in this study for the isolation and electrophysiology of isolated sino-atrial node myocytes of the rabbit have been outlined previously (DiFrancesco et al., 1986). Cells were allowed to settle in Petri dishes, and were superfused with normal Tyrode solution containing (mM): NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; D-glucose, 5.5; Hepes–NaOH, 5; pH=7.4. All procedures were carried out in accordance with the Declaration of Helsinki.

2.2. Electrophysiology

In macro-patch experiments the temperature was kept at 27–28 °C and the patch-pipette solution contained (mM): NaCl, 70; KCl, 70; $CaCl_2$ 1.8; $MgCl_2$, 1; $BaCl_2$, 1; $MnCl_2$, 2; Hepes–KOH, 5; pH=7.4. The control solution perfusing the intracellular side of the membrane patches contained (mM): K–aspartate, 130; NaCl, 10; $CaCl_2$, 2; EGTA, 5 and Hepes–KOH, 10; pH=7.2, pCa=7. In some experiments, the calcium concentration of the bath solution was reduced to 0.1 nM according to the calculation of Fabiato and Fabiato (1979) and the correction of Tsien and Rink (1980).

Macro-patches containing hundreds of f-channels were formed using a large-tipped pipette (0.5 to 2 MΩ) as previously described (DiFrancesco and Tortora, 1991). W7 (*N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) (Calbiochem) and calmodulin (Calbiochem) were dissolved in either distilled water and ethanol (50/50) or distilled water, respectively, divided into aliquots, and stored at −20 °C until use. Ethanol was added to control solutions at the same concentration used in test solutions (lower than 0.1%).

2.3. Data analysis

The time course of macro-patch I_f current under the influence of the modifying compounds was recorded by applying hyperpolarizing steps of 3 s duration at a frequency of 1/15 Hz. At steady-state, the voltage dependence of I_f was described by the equation: $I_f(E)=g_f(E)*(E-vE_f)=g_{fmax}*y_\infty(E)*(E-E_f)$, where g_f is the conductance, g_{fmax} the fully-activated conductance, y_∞ (E) the steady-state activation parameter and E_f the reversal potential (DiFrancesco and Noble, 1985). We measured steady-state I/V curves by applying 1 min long hyperpolarizing voltage ramps with a rate of −115 mV/min from a holding potential of −35 mV. Conductance–voltage ($g_f(E)$) relations were then obtained from the above equation as ratios between steady-state I–V curves ($i_f(E)$) and $E-E_f$, where E_f was set to −12.24 mV (DiFrancesco and Mangoni, 1994). Conductance curves were fitted by Boltzmann function $g_f(E)=g_{fmax}*y_\infty$ $(E)=g_{fmax}*1/(1+\exp(E-E_{1/2})/p)$ where $E_{1/2}$ is the half-maximal voltage of activation and p is the inverse-slope factor. This allowed estimation of the shifts of the voltage dependence of conductance (i.e. of the activation parameter y_∞) measured as changes in $E_{1/2}$. Shifts of the I_f activation curve caused by cAMP were also determined by a quicker method not requiring measurement of the conductance–voltage relation (Accili and DiFrancesco, 1996). Shifts were obtained by applying hyperpolarizing steps from −35 mV to near the mid-point of the I_f activation curve and adjusting the holding potential (−35 mV in the control solution) until the cAMP change in I_f was compensated and the control I_f magnitude fully restored. Since the compensation involved a change of the test voltage (from E to $E+s_m$, where s_m is the measured displacement of the holding potential in mV), a correction was introduced to obtain the shift of the activation curve (s, mV), according to the relation: $s=s_m*(1+(y_\infty/(dy_\infty/dE))/(E-E_f))$. In this calculation we used $y_\infty=0.5$ and $dy_\infty/dE=0.10811$ mV^{-1} (see Accili and DiFrancesco, 1996 for details).

When comparing different sets of data, statistical analysis was performed with either the Student's t test or analysis of variance (ANOVA). Values of $P<0.05$ were considered significant. Statistical data were given as mean±S.E.M values.

3. Results

3.1. cAMP binding of f-channel is not directly modulated by Ca^{2+}–calmodulin

To determine the possible direct action of calmodulin on the f-channel, and the functional interaction between calmodulin and cAMP, the f-current was recorded in cell-free inside–out macro-patches. I_f was activated by 3 s hyperpolarizing steps ranging from −35 to −120 mV every 15 s. As expected, the perfusion of cAMP (1 μM) to the intracellular side instantly increased the f-current amplitude by shifting the current to less negative voltages (Fig. 1A). The voltage shift value, evaluated by correcting the current changes with manual compensation of the holding potential (see Methods) was estimated at 9 mV.

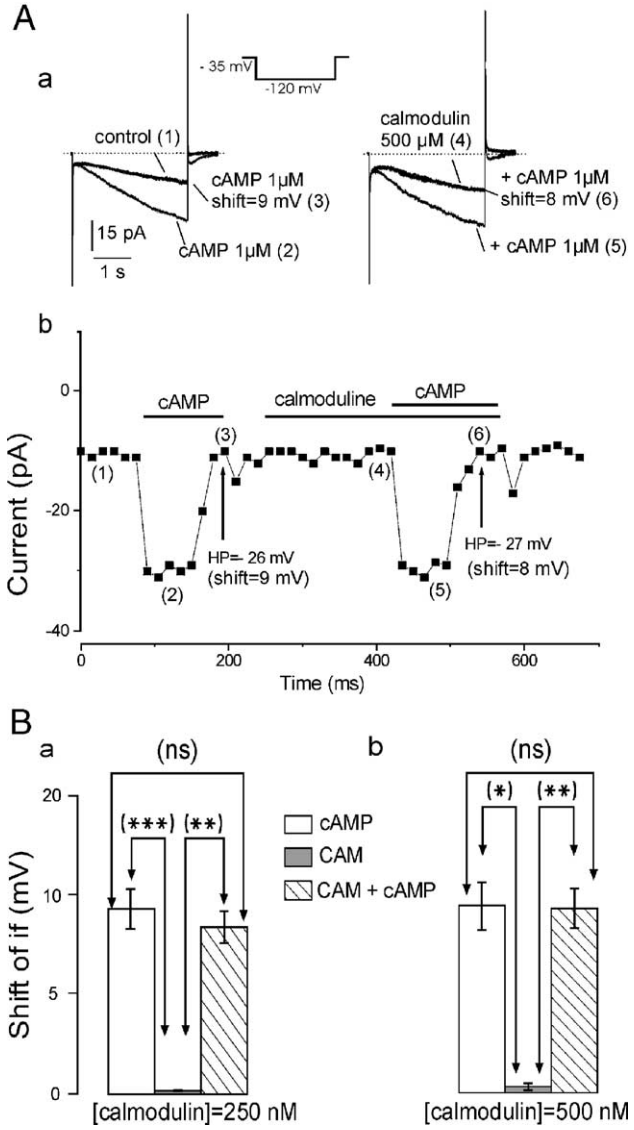

Fig. 1. Action of calmodulin on I_f in inside–out macro-patch, in absence or presence of intracellular cAMP. I_f was activated by a voltage step to −120 mV applied every 15 s from a holding potential of −35 mV (see insert). Intracellular pCa=7. Aa: I_f records during hyperpolarizing steps to the voltages shown taken at various times as indicated in Ab. Ab: Time course of I_f amplitude (start-current subtracted from end-current) during perfusion of the various different solutions (bars). The shift was estimated by the holding potential compensation (see Methods). B: Average shift induced by 1 μM cAMP in absence or presence of two calmodulin concentrations, 250 nM (a) and 500 nM (b) in 6 and 5 cells, respectively. (ns=non-significant, ***P<0.005; **P<0.01 ,*P<0.05). Note that calmodulin failed to change the amplitude and cAMP induced shift.

Following complete washout, subsequent perfusion of the same macro-patch with calmodulin (500 nM), did not significantly change the current amplitude. The simultaneous application of both calmodulin and cAMP induced a current shift to more positive voltages (8 mV) comparable to that obtained in the presence of cAMP alone.

The statistical analysis (Fig. 1B) performed with data from two calmodulin concentrations 250 (Fig. 1Ba) and 500 nM (Fig. 1Bb), in either the absence or presence of 1 μM cAMP, indicated that calmodulin itself was unable to modulate the f-channel activity and the sensitivity of the cAMP binding site. The estimated shifts induced by cAMP perfusion obtained before and during 500 nM calmodulin application were not significantly different (9.21 mV±1.98 vs. 9.3±1.88). These values can be compared to those reported by DiFrancesco and Tortora (1991) and Renaudon et al. (1998) on the cAMP dose–response relationship.

3.2. W7 (Ca^{2+}–calmodulin inhibitor) directly reduces the f-current

Using the perforated patch voltage-clamp technique in guinea-pig sino-atrial node cells, Rigg et al. (2003) showed that the f-current was sensitive to the calmodulin inhibitor W7. These authors suggested that calmodulin was involved in the regulation of the f-current because W7 decreased the pacemaker current by shifting its conductance–voltage curve to more negative voltages. To investigate whether the negative shift of the activation curve could result from a direct binding of W7 on the f-channel, the calmodulin antagonist was tested in inside–

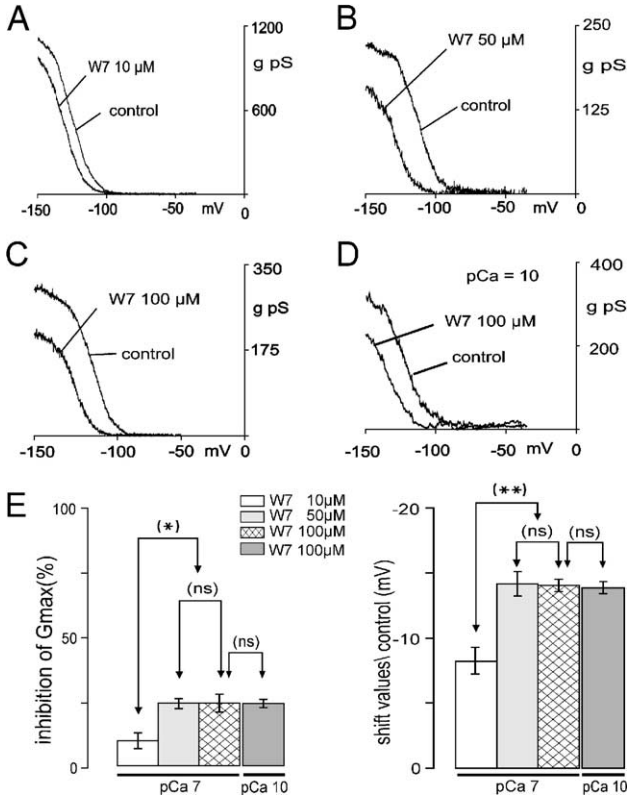

Fig. 2. Action of W7 and/or cAMP on the conductance–voltage relation (g_f (V)), obtained in inside-out macro-patch. $g_f(V)$ curves obtained as detailed in Methods. Effects of intracellular application of 10 μM (A), 50 μM (B) and 100 μM W7 (C) at pCa 7. Boltzmann fitting yielded the following values: (A) $E_{1/2}$=−124 and −132 mV, p=7.3 and 6.9, g_{fmax}=1148 and 1020 pS in control and 10 μM W7, respectively. (B) $E_{1/2}$=−113.8 and −127.5 mV, p=7.8 and 6.8, g_{fmax}=225 and 165 pS in control and 50 μM W7, respectively. (C) $E_{1/2}$=−119 and −134 mV, p=7.8 and 7.2, g_{fmax}=290 and 210 pS in control and 100 μM W7, respectively. (D) Effect of 100 μM W7 in conductance–voltage curve at pCa 10; $E_{1/2}$=−120.1 and −134 mV, p=7.4 and 7.3, g_{fmax}=302 and 221 pS in control and 100 μM W7, respectively. E: Averages g_{fmax} inhibition (left) and $E_{1/2}$ shift (right) induced by 10, 50 and 100 μM W7 at pCa 7 (n=5) and 100 μM W7 at pCa 10 (n=3) (ns=non significant, **P<0.02 ,*P<0.05).

out configuration. The f-current shift was determined with slow ramp protocols (see Methods) before and after W7 application. Note that before the imposition of these slow ramp protocols the reversal potential for I_f was determined by constructing fully-activated current relationships (see DiFrancesco and Mangoni, 1994) in the presence of 100 μM W7 (data not shown). The results show that the drug did not alter the reversal potential compared to the value reported by DiFrancesco and Mangoni (1994) under similar ionic conditions (−13.13±0.62 mV (n=5) vs. −12.24 mV). Fig. 2A, B and C illustrate representative examples of the activation curve for three W7 concentrations in three different cells (10, 50 and 100 μM).

At every concentration, W7 significantly decreased the maximal conductance, and induced a voltage shift towards negative potentials in the position of the current activation curve. Fitting experimental data points by the Boltzmann function (see Fig. 2; legend) indicated that the voltage dependence of the conductance curve shifted by 8 mV ($E_{1/2}$ from −124 to −132 mV), 13.7 mV (from −113.8 to −127.5 mV) and 15 mV (from −119 to −134 mV) after exposure to 10, 50, and 100 μM W7, respectively. The maximal conductance decreases were 11.14%, 26.66% and 27.58% with 10, 50 and 100 μM W7, respectively. Average data are shown in Fig. 2E.

In order to confirm whether the calmodulin antagonist W7 directly inhibited the pacemaker current without accessing calmodulin, W7 was applied to the cytoplasmic side of the patch using a solution containing free-calcium at a very low concentration (pCa=10). The conductance curve (Fig. 2D) obtained in control and in presence of the drug showed that 100 μM W7 reduced the I_f-conductance by 26.8% and induced a shift of the activation curve to more negative potentials estimated at 13.9 mV. Average data summarized in Fig. 1E indicated that both I_f-conductance reduction and negative shift of the current activation curve induced by W7 are not calmodulin dependent.

To evaluate the hypothesis that W7 could affect cAMP binding or that cAMP could alter W7 binding, the effects on the I_f activation curve of sequential intracellular perfusions of 10 μM cAMP, 10 μM W7+10 μM cAMP and 10 μM W7 were investigated. Fitting experimental data points by the Boltzmann function (see Fig. 3 legend) indicated that f-conductance curve shifted by 13.4 mV ($E_{1/2}$ from −127.6 to −114.2 mV), 6.3 mV (from −127.68 to −121.3 mV) and −7.4 mV (from −127.6 to −135 mV) after perfusion with 10 μM cAMP, 10 μM cAMP+10 μM W7, and 10 μM W7, respectively. The positive shift of the I_f-activation curve induced by cAMP was comparable in the absence (13.4 mV) and in the presence of W7 (13.7 mV=6.3+7.4 mV). Similarly, W7 provoked a left shift of the activation curve, which was identical in the absence (7.4 mV) and in the presence (7.1 mV) of cAMP. As it has been observed in this study W7 at 10 μM reduced the maximal conductance by approximately 11.31% and 10.22% in the presence of 10 μM cAMP.

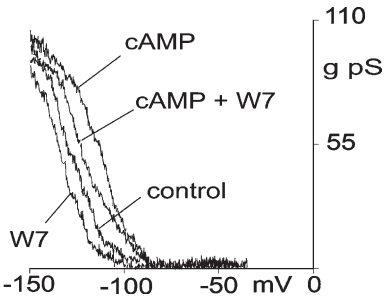

Fig. 3. Effect of W7 on conductance–voltage relation in the absence and in the presence of cAMP. $g_f(V)$ relation in after sequential perfusion control, 10 μM cAMP, 10 μM W7+10 μM cAMP and 10 μM W7 (return was omitted for clarity) as indicated. $E_{1/2}$=−127.6, −114.2, −121.3 and −135 mV, respectively.

This effect was also obtained with sequential intracellular perfusions of 10 μM W7, 10 μM cAMP+10 μM W7 and 10 μM cAMP, suggesting an independent effect of the two molecules. Similar findings were observed in all four cells studied.

4. Discussion

The present data, obtained in inside–out macro-patch configuration in rabbit sino-atrial cells, clearly show that the f-channel is not directly modulated by Ca^{2+}–calmodulin in cardiac pacemaker cells. In contrast to the cyclic nucleotide-gated channel characterized in the olfactory receptor (Kaupp, 1991), the apparent affinity of the f-channel cyclic nucleotide-binding site for cAMP is not reduced in the presence of Ca^{2+}–calmodulin. These data exclude the calmodulin-binding site or a site that couples calmodulin binding to channel gating or on an adjacent protein. An absence of a direct effect of Ca^{2+}–calmodulin on I_f is also indicated by previous data from experiments using whole cell, showing that calmidazolium (calmodulin antagonist) did not affect I_f (Hagiwara and Irisawa, 1989). These results differ from those of Rigg et al. (2003) reported in guinea-pig sino-atrial node cells. Using the perforated patch-clamp technique, these authors detected a large reduction in the pacemaker current amplitude after the perfusion of different calmodulin antagonists; in particular a well-known antagonist, W7. In this study, 10 μM W7 was reported to induce a significant shift of the activation curve of I_f to a more negative potential without affecting the slope of the activation curve. In contrast to the interpretation of Rigg et al., we consider that this negative shift (estimated at 11 mV) was accompanied by a small decrease in the maximal conductance (see Fig. 2, Rigg et al., 2003). These data obtained from experiments using whole-cell correspond to those of the current study, using a more direct approach. Indeed, in the inside–out membrane macro-patch configuration we demonstrate that exposure to 10 μM W7 caused a negative shift in the voltage activation of I_f (estimated at 8.2±1.9 mV) and slightly reduced the I_f conductance without altering the slope factor. Both effects depend on the W7 concentration, with maximal effect at 50 μM. Furthermore, the experiments conducted using free-Ca^{2+} at low concentrations on the cytoplasmic side of the patch (pCa=10) indicated that W7 effects are calmodulin independent. Thus, W7 seems to act on I_f in a membrane-delimited manner directly on the inside of the channel or on a regulatory factor in the membrane closely related to the channel. These experimental observations are at odds with the hypothesis of Rigg et al. (2003), who suggested that the W7 effect involves the calmodulin pathway. These

authors made the assumption that the inhibition of calmodulin would decrease the activation of calcium-stimulated adenylyl cyclase, which would then lower intracellular cAMP levels and thus would shift the f-current activation curve to more negative potentials. Nonetheless, as shown previously (Cali et al., 1994; Defer et al., 2000), the Ca^{2+}–calmodulin sensitive adenylyl cyclases (AC1 and AC8) are mainly expressed in the central nervous system and are absent in the heart. Evaluation of the effect of W7 on the Ca^{2+}–calmodulin interaction with adenylyl cyclases requires further investigation. On the other hand, whatever the effect of W7 on adenylyl cyclase, the present results indicate that W7 directly inhibits f-channels. As reported for olfactory cyclic nucleotide-activated currents (Kleene, 1994), we propose a possible interaction of W7 with the nucleotide binding site located in the C-terminal region of the f-channel. However, f-channel modulation by cAMP seems to be unaltered by W7 (Fig. 2D), suggesting the presence of different binding sites. As described for the C-terminal cyclic nucleotide-binding domain, the W7 site could modulate S6 helix of the channel thought to form the activation gate in HCN channel (Wainger et al., 2001). Finally, the effect of W7 on the activation characteristics of I_f may reflect either a decrease in channel conductance and/or a reduced probability of opening. A number of organic compounds have been described that specifically block I_f, e.g. include ZD-7288 (BoSmith et al., 1993), UL-FS49 (Zatebradine) (Van Bogaert and Goethals, 1992), and S-16257 (Ivabradine) (Bois et al., 1996). Among these drugs, the blocking properties of ZD7288 are similar to those of W7. Indeed, this bradycardic agent directly shifted the I_f activation curve and reduced the activation curve amplitude in guinea-pig dissociated sino-atrial node cells (BoSmith et al., 1993). The different effects of W7 indicate the drug's action on voltage sensor mechanisms and/or in the pore of the f-channel. In sino-atrial node cells, HCN4 represents the predominantly expressed HCN channel isoform (Ishii et al., 2001; Moosmang et al., 2001; Altomare et al., 2003). Thus, it seems reasonable to conclude, that the HCN4 isoform in cardiac pacemaker cells is not directly regulated by calmodulin. Although W7 is usually considered as a selective calmodulin antagonist, we show that it is able to directly block the pacemaker current of heart sino-atrial node cells. Analogues of W7 could be synthesized with higher potency and greater selectivity and could constitute a new family of bradycardic agents.

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