INTRODUCTION

Beat-to-beat variability of action potential duration (also called short term variability, SV) is an intrinsic property of various in vivo and in vitro mammalian cardiac preparations including the human heart (1-3). Although SV is considered one of the best proarrhythmic predictors (4-6), its exact mechanism is still not fully understood. Involvement of several factors, such as stochastic gating of various ion channels (7, 8), intensity of cell-to-cell coupling (9), action potential morphology (10) or stimulation frequency (11), in modulation of SV have already been implicated. Since many cardiac ion currents are influenced by intracellular calcium concentration ([Ca^{2+}]i), we decided to investigate the effects of [Ca^{2+}]i changes on SV in the present study under two experimental conditions: (1) [Ca^{2+}]i was manipulated in an unspecified manner using Ca^{2+} ionophore and Ca^{2+} chelator agents, and (2) the role of SR-mediated [Ca^{2+}]i release was tested using ryanodine and cyclopiazonic acid - both are known to diminish [Ca^{2+}]i release from the SR. All the experiments were performed in canine ventricular myocytes, because this preparation is believed to resemble human ventricular cells regarding their action potential morphology and kinetics of the underlying ion currents (12, 13), and also due to the significant amount of experimental data on SV accumulated already in dogs. The results indicate that relative SV is increased by elevation of [Ca^{2+}]i, where contribution of the SR-related [Ca^{2+}]i release seems to be dominant. This finding may help better understanding the mechanisms of [Ca^{2+}]i-related arrhythmogenesis.

MATERIALS AND METHODS

Isolation of single canine ventricular myocytes

Adult mongrel dogs of either sex were anaesthetized with intramuscular injections of 10 mg/kg ketamine hydrochloride (Calyposol, Richter Gedeon, Hungary) + 1 mg/kg xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, The Netherlands) according to protocols approved by the local ethical committee (license No: 18/2012/DEMAB) in line with the ethical standards laid down in the Declaration of Helsinki in 1964 and its later amendments. The hearts were quickly removed and placed in Tyrode solution. Single myocytes were obtained by enzymatic dispersion using the segment perfusion technique, as described previously (14). Briefly, a wedge-shaped section of...
the left ventricular wall supplied by the left anterior descending coronary artery was dissected, cannulated and perfused with oxygenized Tyrode solution. After removal of blood the perfusion was switched to a nominally Ca\(^{2+}\)-free Joklik solution (Minimum Essential Medium Eagle, Joklik Modification, Sigma) for 5 min. This was followed by 30 min perfusion with Joklik solution supplemented with 1 mg/ml collagenase (Type II. Worthington, Chemical Co.) and 0.2 % bovine serum albumin (Fraction V., Sigma) containing 50 μM Ca\(^{2+}\). After gradually restoring the normal external Ca\(^{2+}\) concentration, the cells were stored in Minimum Essential Medium Eagle until use. Drugs were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) except ryanodine, which was purchased from Cayman Chemical Company (Michigan, USA).

**Recording of action potentials**

All electrophysiological measurements were performed at 37°C. The rod-shaped viable cells showing clear striation were sedimented in a plexiglass chamber of 1 ml volume allowing continuous superfusion (at a rate of 2 ml/min) with modified Krebs solution gassed with a mixture of 95% O\(_2\) and 5% CO\(_2\) at pH = 7.4. The modified Krebs solution contained (in mM): NaCl 128.3; NaHCO\(_3\), 21.4; KCl, 4.0; CaCl\(_2\), 1.8; MgCl\(_2\), 0.42; and glucose 10. Transmembrane potentials were recorded using 3 M KCl filled sharp glass microelectrodes having tip resistance between 20 and 40 MΩ. These electrodes were connected to the input of Multiclamp 700A, 700B or Axoclamp-2B amplifiers (Molecular Devices, Sunnyvale, CA, USA). The cells were paced through the recording electrode at steady cycle length of 1 s using 1–2 ms wide rectangular current pulses having amplitudes of 20 and 500 ms) in a way not related to specific interaction with one or another specific ion current. The results are presented in Fig. 1, where SV obtained with any given current pulse was plotted against the corresponding APD. Similarly, the current-induced changes in SV and APD (ΔSV and ΔAPD, respectively) were also plotted, and the data were fitted to an exponential function yielding the solid curves in Fig. 1C and 1D (the equations and the estimated parameters will be discussed later in Fig. 4). This approach allows to study the effect of any drug or intervention on relative SV directly, since a data point above the curve is a marker of an increased, while below the curve of a reduced relative SV.

**Effects of unspecific changes in [Ca\(^{2+}\)] in cell shortening**

Since in the following sections the effects of BAPTA-AM, A23187, ryanodine and cyclopiazonic acid on relative SV are studied, the effects of these agents on unloaded cell shortening, considered as an indicator of [Ca\(^{2+}\)] changes, had to be first monitored. Drugs were superfused after reaching steady-state amplitudes of shortening. According to Fig. 2, cells shortening was suppressed by all of the drugs studied. While in the case of BAPTA-AM, ryanodine and cyclopiazonic acid the decreased cell length approximated the diastolic level, indicating a suppressed calcium release, the enveloping curve of the cell shortening was close to the systolic level in the presence of A23187. This is in line with the calcium accumulation expected in the presence of a calcium ionophore.

**Effects of unspecific changes in [Ca\(^{2+}\)], on relative short term variability**

Unspecific elevation and reduction of [Ca\(^{2+}\)], was based on the simple assumption that if the cell is loaded directly with Ca\(^{2+}\), it must increase [Ca\(^{2+}\)], and conversely, [Ca\(^{2+}\)], must be reduced by an intracellularly applied Ca\(^{2+}\) chelator. Therefore, the cells
were exposed to the Ca\(^{2+}\) ionophore A23187 (1 µM for 25 min) or they were loaded with the cell-permeant acetoxy-methylester form of the Ca\(^{2+}\) chelator BAPTA (5 µM BAPTA-AM was applied for 25 min). As demonstrated in Fig. 3A, 3C, 3F and 3G, 1 µM A23187 significantly increased relative SV (shortening of APD with unchanged SV). Interestingly, an absolute increase in SV was also observed at the beginning of superfusion with A23187. In contrast, loading the cells with BAPTA-AM caused a significant reduction in relative SV primarily due to the progressive lengthening of APD as more and more intracellular BAPTA was released by the esterases (Fig. 3B, 3D, 3E and 3G). Reduction of SV was not significant statistically - except for the 15th min superfusion with BAPTA-AM, where the decrease in SV was significant. In summary, relative SV was increased by elevation of cytosolic Ca\(^{2+}\) concentration.

The effect of BAPTA-AM was further investigated by combining current injections with BAPTA-AM pretreatment. In these experiments action potentials were prolonged or shortened with the application of appropriately tailored depolarizing or hyperpolarizing pulses, respectively. Thus the SV-APD relationship could be determined in the presence of BAPTA-AM and compared to that obtained under control conditions. According to the results presented in Fig. 4A and 4B, the time constant (t values) obtained by fitting the individual data points

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**Fig. 1.** Dependence of beat-to-beat variability on the duration of action potentials. (A), (B): Superimposed sets of representative action potentials recorded from myocytes after injection of outward (-300 pA) and inward (+50 pA) current pulses, and the corresponding Poincare plots. Each panel represents a set of 50 consecutive action potentials. (C): SV plotted against the corresponding APD. Both parameters were simultaneously modified using constant current pulses with amplitudes varying from -500 to +70 pA, as specified at the bottom of the figure. (D): Data from the same experiments (9 cells from 8 dogs). Here the changes of SV and APD induced by each current pulse (ΔSV and ΔAPD, respectively) were plotted. Open circles and bars denote mean ± S.E.M. values, filled circles indicate controls. Solid curves were obtained by fitting data to monoexponential functions in order to determine the SV vs. APD and ΔSV vs. ΔAPD relationships. These curves are used as references in the further studies when evaluating drug effects on relative SV.
Fig. 2. Time dependent effects of BAPTA-AM (5 µM, 7 cells from 3 dogs, (A), (B)), A23187 (1 µM, 5 cells from 3 dogs, (C), (D)), ryanodine (10 µM, 5 cells from 3 dogs, (E), (F)) and cyclopiazonic acid (CPA, 1 µM, 4 cells from 3 dogs, (G), (H)) on unloaded cell shortening. Representative experiments are displayed in the left panels, while the average data are presented in right panels. Drugs were applied after reaching steady-state amplitudes of cell shortening. Relative cell length was normalized to diastolic cell length obtained in the last 1 min period before drug-application (100%). Cell shortening was expressed in percent of diastolic cell length. Symbols and bars are means ± S.E.M., arrows and dashed lines indicate time of drug-administration.
to a monoexponential function were much longer in BAPTA-AM than in control, indicating that relative SV is reduced by BAPTA-AM at any APD value, however, this reduction was greater and more prominent at longer APDs.

The effect of BAPTA-AM on relative SV was studied also when APD was pharmacologically lengthened (Fig. 4C). The rapid delayed rectifier K⁺ current (I_{Kr}) was suppressed by 100 nM dofetilide or 200 nM E-4031 (16), while 100 nM veratridine and 200 nM BAY K8644 were used to increase late Na⁺ current (I_{Na-late}) and L-type Ca²⁺ current (I_{Ca}) respectively (17, 18). Although the effects of BAPTA-AM superfusion were different when applying after I_{Kr}-blockade and following enhancement of inward currents, the reduction of relative SV following the chelation of [Ca²⁺]i by BAPTA-AM was a common observation in these studies - independently of the actual experimental conditions.

Contribution of sarcoplasmic reticular Ca²⁺ release

Contribution of transient changes of [Ca²⁺]i due to Ca²⁺ released from the sarcoplasmic reticulum was studied using ryanodine and cyclopiazonic acid (Fig. 5). The former blocks Ca²⁺ release from the SR at 10 µM concentration by decreasing the open probability of the Ca²⁺ release channel in the SR membrane (19). Cyclopiazonic acid is a selective inhibitor of the SR Ca²⁺ pump resulting in a depletion of the SR Ca²⁺ release pool (20, 21). As shown in Fig. 5G, application of these agents resulted in a comparable reduction of relative SV. Ryanodine caused a transient decrease in SV with no significant change in APD (Fig. 5A, 5C and 5E), while 1 µM cyclopiazonic acid decreased SV significantly, accompanied with a small, statistically not significant reduction of APD (Fig. 5B, 5D and 5F). These results clearly indicate that systolic Ca²⁺ release, experienced by the surface membrane, is a relevant signal for SV modulation.

Role of the Na⁺-Ca²⁺ exchange

The influence of the Na⁺-Ca²⁺ exchanger (NCX) on SV was studied after inhibition of the exchanger by SEA0400. This agent is thought to be a selective blocker of NCX, when applied at a sufficiently low concentration of 300 nM (22). Exposure of the cells to 300 nM SEA0400 for 25 min increased SV and decreased APD significantly, clearly resulting in a marked enhancement of relative SV (Fig. 6).
In the present study we have shown that relative SV was increased by elevation of \([\text{Ca}^{2+}]_i\) and decreased by its reduction. This was true practically independently of the actual experimental conditions. Best demonstration of this finding is in Fig. 4C, where chelation of \([\text{Ca}^{2+}]_i\) by BAPTA-AM decreased relative SV in all cases. This reduction in relative SV was mainly due to an actual decrease in SV when BAPTA-AM was applied following \(I_{Kr}\) blockade by dofetilide or E-4031. Under these conditions APD was shortened by BAPTA-AM. On the other hand, when APD was lengthened by veratridine or BAY K8644 (due to enhancement of \(I_{Na\\text{late}}\) and \(I_{Ca\\text{,late}}\) respectively), as well as under control conditions, relative SV was decreased by BAPTA-AM due to the marked prolongation of APD. The BAPTA-AM induced prolongation was likely the consequence of a direct blocking effect of BAPTA-AM on \(I_{Kr}\) (23). Although the underlying ionic mechanism was basically different under these conditions (i.e. the set of operating \(\text{Ca}^{2+}\)-sensitive ion channels were likely different), reduction of \([\text{Ca}^{2+}]_i\) decreased relative SV uniformly.

More importantly, the contribution of the SR-related \([\text{Ca}^{2+}]_i\) release seems to be a dominant factor in \(\text{Ca}^{2+}\)-sensitive modulation of SV. Ryanodine, the blocker of the SR \(\text{Ca}^{2+}\) release channel (19) decreased SV directly. Cyclopiazonic acid, the selective inhibitor of the SERCA \(\text{Ca}^{2+}\) pump resulting in a depletion of the SR \(\text{Ca}^{2+}\) release pool (20, 21) caused similar effect. Both agents are known to decrease the amplitude of the intracellular calcium transient in cardiac cells (24, 25), and in line with these results, they decreased unloaded cell shortening in our experiments. Although the time-dependent pattern of changes in SV and APD were different, the resultant reduction in relative SV was similar with both drugs. The biphasic effect of \(A_23187\) on SV suggests that probably more than one mechanism is involved in the effect of \([\text{Ca}^{2+}]_i\) on SV (26). These observations are in accordance with the results of Johnson et al. (27) demonstrating the increment of SV during diastolic spontaneous \([\text{Ca}^{2+}]_i\), release induced by \(\beta\)-adrenergic stimulation. The important role of \([\text{Ca}^{2+}]_i\) transients in modulation of SV supports the view that it is manifested in fact by the \(\text{Ca}^{2+}\)-sensitive ion channels in the surface membrane, which are

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**DISCUSSION**

In the present study we have shown that relative SV was increased by elevation of \([\text{Ca}^{2+}]_i\), and decreased by its reduction. This was true practically independently of the actual experimental conditions. Best demonstration of this finding is in Fig. 4C, where chelation of \([\text{Ca}^{2+}]_i\) by BAPTA-AM decreased relative SV in all cases. This reduction in relative SV was mainly due to an actual decrease in SV when BAPTA-AM was applied following \(I_{Kr}\) blockade by dofetilide or E-4031. Under these conditions APD was shortened by BAPTA-AM. On the other hand, when APD was lengthened by veratridine or BAY K8644 (due to enhancement of \(I_{Na\\text{late}}\) and \(I_{Ca\\text{,late}}\), respectively), as well as under control conditions, relative SV was decreased by BAPTA-AM due to the marked prolongation of APD. The BAPTA-AM induced prolongation was likely the consequence of a direct blocking effect of BAPTA-AM on \(I_{Kr}\) (23). Although the underlying ionic mechanism was basically different under these conditions (i.e. the set of operating \(\text{Ca}^{2+}\)-sensitive ion channels were likely different), reduction of \([\text{Ca}^{2+}]_i\) decreased relative SV uniformly.

More importantly, the contribution of the SR-related \([\text{Ca}^{2+}]_i\) release seems to be a dominant factor in \(\text{Ca}^{2+}\)-sensitive modulation of SV. Ryanodine, the blocker of the SR \(\text{Ca}^{2+}\) release channel (19) decreased SV directly. Cyclopiazonic acid, the selective inhibitor of the SERCA \(\text{Ca}^{2+}\) pump resulting in a depletion of the SR \(\text{Ca}^{2+}\) release pool (20, 21) caused similar effect. Both agents are known to decrease the amplitude of the intracellular calcium transient in cardiac cells (24, 25), and in line with these results, they decreased unloaded cell shortening in our experiments. Although the time-dependent pattern of changes in SV and APD were different, the resultant reduction in relative SV was similar with both drugs. The biphasic effect of \(A_23187\) on SV suggests that probably more than one mechanism is involved in the effect of \([\text{Ca}^{2+}]_i\) on SV (26). These observations are in accordance with the results of Johnson et al. (27) demonstrating the increment of SV during diastolic spontaneous \([\text{Ca}^{2+}]_i\), release induced by \(\beta\)-adrenergic stimulation. The important role of \([\text{Ca}^{2+}]_i\) transients in modulation of SV supports the view that it is manifested in fact by the \(\text{Ca}^{2+}\)-sensitive ion channels in the surface membrane, which are
known to directly experience $[\text{Ca}^{2+}]_{i}$ changes in the narrow submembrane compartment, also known as fuzzy space.

In line with this approach, SEA0400, the potent inhibitor of NCX strongly increased relative SV. NCX is believed to be the main mechanism of $\text{Ca}^{2+}$ extrusion from the intracellular space in cardiac cells (28). Suppression of this component of $\text{Ca}^{2+}$ elimination is expected to increase $[\text{Ca}^{2+}]_{i}$ beneath the sarcolemma, which effect was well documented in rat myocytes (29). Although no significant increase in $[\text{Ca}^{2+}]_{i}$ could be detected in canine cells on exposure to SEA0400 when measuring $[\text{Ca}^{2+}]_{i}$ in the bulk phase of the cytosol (30), it is likely that suppression of NCX results in an elevated $[\text{Ca}^{2+}]_{i}$ in the submembrane compartment of canine myocytes as well. Indeed, selective suppression of NCX by SEA0400 increased SV while APD was reduced. Both changes were likely consequences of the elevated submembrane $\text{Ca}^{2+}$ concentration. It is worthy of note that under markedly different experimental conditions (i.e. after prolongation of APD and development of early afterdepolarizations by dofetilide in hypertrophied remodeled cells), 1 µM SEA0400 decreased the short term variability of APD and suppressed early afterdepolarizations, while the elevated amplitude of $[\text{Ca}^{2+}]_{i}$ transient was also decreased by SEA0400 (31). Although the effects of NCX inhibitors on $[\text{Ca}^{2+}]_{i}$ are strongly dependent on experimental conditions (32), reduction of $[\text{Ca}^{2+}]_{i}$ released from the SR was accompanied by a decreased SV, supporting further our conclusions.

Regarding the underlying mechanisms (i.e. which $\text{Ca}^{2+}$ sensitive ion current may be responsible for the $\text{Ca}^{2+}$ sensitivity of SV), it has been shown that there are 3 ion currents in canine myocytes which may have a larger impact on beat-to-beat variability, these are: late $I_{\text{Na}}$, $I_{\text{Kr}}$ and $I_{\text{Ca}}$ (10, 15). Although activation of $I_{\text{Na}}$ or suppression of $I_{\text{Kr}}$ were reported to increase SV drastically (10, 15), these alterations cannot be easily deduced from elevation of $[\text{Ca}^{2+}]_{i}$. The third current is $I_{\text{Ca}}$, which is suppressed in amplitude by high $[\text{Ca}^{2+}]_{i}$ due to its $\text{Ca}^{2+}$ dependent inactivation (33). Indeed, relative SV was reduced by BAY K8644 and increased by nisoldipine, indicating that $I_{\text{Ca}}$ is an important regulator of SV under normal conditions (15) as well as in $\text{Ca}^{2+}$ overloaded cells (27). In conclusion, the major effect of $[\text{Ca}^{2+}]_{i}$ on SV is likely to occur via reduction of $I_{\text{Ca}}$ by enhancing its $\text{Ca}^{2+}$ dependent inactivation, however, minor contribution of other $\text{Ca}^{2+}$ sensitive ion currents, such as $I_{\text{NCX}}$, $I_{\text{K}}$, and a variety of $\text{Ca}^{2+}$ sensitive K$^{-}$ currents, cannot fully be ruled out.

Fig. 5. Effects of sarcoplasmic reticular calcium release on beat-to-beat variability. Cells were exposed to ryanodine (10 µM, (A), (C)), or cyclopiazonic acid (CPA, 1 µM, (B), (D)), then the drug-induced changes in SV and APD were analyzed as a function of time (E), 8 cells from 5 dogs and (F), 9 cells from 6 dogs). Superimposed sets of action potentials (A), (B), Poincare plots obtained at 25 min of superfusion (C), (D) and average results (E), (F) are displayed. Relative SV data ($\Delta$SV vs. $\Delta$APD) obtained after 15 min exposure to ryanodine and CPA are presented in panel (G). Symbols and bars are means ± S.E.M., asterisks denote significant differences from control ($P<0.05$), and the solid curve in panel (G) indicates the $\Delta$SV-$\Delta$APD relationship obtained in control with current injections.
It is generally accepted that elevation of [Ca\(^{2+}\)], is highly proarrhythmic (34). High [Ca\(^{2+}\)]-induced arrhythmias are generated mainly by two mechanisms: elevation of [Ca\(^{2+}\)], causes uncoupling of myocytes due to closure of gap junctions, resulting in increased longitudinal resistance, slower conduction, and ultimately re-entry. The second - not less important - mechanism is development of delayed afterdepolarizations, mediated by Ca\(^{2+}\)-overloaded SR. Based on the present results a third potential Ca\(^{2+}\) dependent proarrhythmic mechanism has to be considered: the increased beat-to-beat variability of APD. Further studies are required to assess the actual significance of the increased beat-to-beat variability, induced by high [Ca\(^{2+}\)], in arrhythmogenesis.

Acknowledgements: Financial support was provided by grants from the Hungarian Scientific Research Fund (OTKA-K100151, OTKA-K109736, OTKA-K101196, OTKA-PD101171 and OTKA-NK104331). Further support was obtained from the Hungarian Government and the European Community (TAMOP-4.2.2.A-11/1/KONV-2012-0045). Research of KK and BH was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TAMOP-4.2.4.A/2-11/1-2012-0001 ‘National Excellence Program’. The authors thank Miss Eva Sagi for excellent technical assistance.

Conflict of interests: None declared.

Fig. 6. Effect of 300 nM SEA0400 on beat-to-beat variability. (A), (B): Superimposed sets of action potentials and Poincare plots obtained in control and after exposure to 300 nM SEA0400 for 25 min. Average APD and SV results obtained under control conditions and in the presence of SEA0400 (7 cells from 3 dogs) are presented in panel (C), while the SEA0400-induced changes (AAPD and ΔSV) in panel (D). Columns and bars are means ± S.E.M., asterisks denote significant changes induced by SEA0400 (P<0.05).

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Received: September 8, 2014
Accepted: January 16, 2015

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