Revisiting the ionic mechanisms of early afterdepolarizations in cardiomyocytes: predominant by Ca waves or Ca currents?

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Zhao Z, Wen H, Fefelova N, Allen C, Baba A, Matsuda T, Xie LH. Revisiting the ionic mechanisms of early afterdepolarizations in cardiomyocytes: predominant by Ca waves or Ca currents? Am J Physiol Heart Circ Physiol 302: H1636–H1644, 2012. First published February 3, 2012; doi:10.1152/ajpheart.00742.2011.—Early afterdepolarizations (EADs) have been implicated in severe cardiac arrhythmias and sudden cardiac deaths. However, the mechanism(s) for EAD genesis, especially regarding the relative contribution of Ca2+ wave (CaW) vs. L-type Ca current (I\textsubscript{Ca,L}), still remains controversial. In the present study, we simultaneously recorded action potentials (APs) and intracellular Ca2+ images in isolated rabbit ventricular myocytes and systematically compared the properties of EADs in the following two pharmacological models: 1) hydrogen peroxide (H2O2; 200 μM); and 2) isoproterenol (100 nM) and BayK 8644 (50 nM) (Iso + BayK). We assessed the rate dependency of EADs, the temporal relationship between EADs and corresponding CaWs, the distribution of EADs over voltage, and the effects of blockers of I\textsubscript{Ca,L}, Na/Ca exchangers, and ryanodine receptors. The most convincing evidence came from the AP-clamp experiment, in which the cell membrane clamp was switched from current clamp to voltage clamp using a normal AP waveform without EAD; CaWs disappeared in the H2O2 model, but persisted in the Iso + BayK model. We postulate that, although CaWs and reactivation of I\textsubscript{Ca,L} may act synergistically in either case, reactivation of I\textsubscript{Ca,L} plays a predominant role in EAD genesis under oxidative stress (H2O2 model), while spontaneous CaWs are a predominant cause for EADs under Ca2+ overload condition (Iso + BayK model).

reactive oxygen species; β-adrenergic stimulation; Ca2+ wave; L-type Ca2+ current; Na/Ca exchanger

EARLY AFTERDEPOLARIZATIONS (EADs), delayed afterdepolarizations (DADs), and triggered activities (TAs) are implicated in arrhythmias and sudden cardiac deaths (4). Although afterdepolarizations and TAs have been extensively studied, their underlying mechanisms remain incompletely understood. While there is a consensus on the role of spontaneous sarcoplasmic reticulum (SR) Ca2+ release and Ca2+ waves (CaWs) in the generation of DADs (36), significant discrepancies exist regarding the mechanisms of EADs. For example, early experimental and computer model studies (14, 19, 44) suggested EADs are exclusively caused by the reactivation of L-type calcium current (I\textsubscript{Ca,L}) without the involvement of CaWs. However, accumulating evidence obtained from recent experimental (33, 40) and computer simulation studies (13) support a mechanism involving CaW for EAD formation under Ca overload conditions. Thus it seems likely that EAD generation can be mediated by at least two mechanisms: 1) sarcolemmal-dependent (or I\textsubscript{Ca,L}-dominant) mechanism: enhancement of inward currents during repolarization, such as I\textsubscript{Ca,L}; and 2) SR-dependent (or CaW-dominant) mechanism: Na/Ca exchange current (I\textsubscript{NCX}) or transient inward current (I\textsubscript{t}) initiated by spontaneous SR Ca2+ release through the ryanodine receptor (RyR) under Ca2+ overload conditions. Furthermore, these two mechanisms (i.e., I\textsubscript{Ca,L} and I\textsubscript{NCX}) are considered to be highly interactive and function synergistically to induce EADs (41). However, disputes still remain on the relative contribution of different mechanisms to EAD generation in different models (9, 31, 40). It is unquestionable that a better understanding of the factors playing a primary role in EAD formation may be helpful for developing therapeutic approaches.

In our previous studies, we have established the following two different EAD models in isolated rabbit ventricular myocytes: 1) hydrogen peroxide (H2O2) model: EADs induced by reactive oxygen species (ROS) and H2O2 (42); and 2) Iso + BayK model: EADs induced by isoproterenol (100 nM) in addition to BayK 8644 (50 nM) (43). Here, to further clarify the mechanisms accounting for EADs under different conditions, we make systematic comparisons between these two types of EADs. We have provided more convincing evidence suggesting that I\textsubscript{Ca,L} reactivation and CaW may account for distinct predominant ionic mechanisms under different conditions, although they may act synergistically to generate EADs.

MATERIALS AND METHODS

Cell isolation. Single ventricular myocytes were enzymatically isolated from adult rabbit hearts. Briefly, the hearts were removed from adult New Zealand White rabbits (2–3 kg), anesthetized with intravenous pentobarbital sodium, and hearts were perfused retrogradely in Langendorff fashion at 37°C with nominally Ca2+-free Tyrode solution containing ~1.4 mg/ml collagenase (type II; Worthington) and 0.1 mg/ml protease (type XIV; Sigma) for 25–30 min. After the enzyme solution was washed out, the hearts were removed from the perfusion apparatus and swirled in a culture dish. The myocytes were isolated from left ventricles without specific separation from different layers. The Ca2+ concentration was slowly increased to 1.8 mM, and the cells were stored at room temperature and used within 8 h. The use and care of the animals in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey–New Jersey Medical School.

Patch-clamp methods. Myocytes were patch clamped using the perforated whole cell configuration of the patch-clamp technique in
the current-clamp or voltage-clamp mode. Recording pipettes (resistance, 2–4 MΩ) were filled with internal solution containing the following (in mM): 110 NaCl, 5 KCl, 10 NaH2PO4, 0.1 EGTA, 5 MgATP, 5 Na3-creatine phosphate, and 0.05 cAMP (pH 7.2, adjusted with KOH). Myocytes were superfused with normal Tyrode solution containing the following (in mM): 136 NaCl, 5.4 KCl, 0.33 Na2HPO4, 1.8 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES (pH 7.4, adjusted with NaOH). Action potentials (APs) were elicited with 2-ms, 2- to 4-nA square pulses at various pacing cycle lengths (PCLs). In some cells, Ca2+ transients/waves were recorded under current-clamp or AP-clamp (voltage-clamp with a fixed AP waveform) conditions. Electrical signals were measured with a MultiClamp 700A patch-clamp amplifier, controlled by a personal computer using a Digidata 1322A interface, driven by pClAMP 10 software.

Intracellular Ca2+ concentration measurement. Myocytes were loaded with the Ca2+ indicator fluo-4 by incubating them for ~30 min in bath solution containing 4 μM fluo-4 AM (Molecular Probe), after which the cells were washed and placed in a heated chamber on an inverted microscope. Intracellular Ca2+ concentration ([Ca2+]i) fluorescence was recorded using an Andor Ixon charge-coupled device camera (Andor Technology) operating at ~100 frame/s with a spatial resolution of 500 × 400 pixels. Fluorescence intensity was measured as the ratio of fluorescence (F) over the baseline diastolic fluorescence (F0).

Chemicals. SEA0400 was synthesized by Taisho Pharmaceuticals (Saitama, Japan). Nifedipine and BayK 8644 were first dissolved in DMSO as stock solutions before being diluted into the bath solution to the final concentration. The maximum DMSO concentration was <0.2% (vol/vol). Chemicals and reagents were purchased from Sigma unless indicated. All experiments in the present study were carried out at 35–37°C.

Statistical analysis. The incidence of EADs within groups of 20 consecutive AP recordings was analyzed by Fisher’s exact test. All other data are shown as means ± SD. Statistical differences were evaluated with Student’s paired or unpaired t-tests. P < 0.05 is considered as statistically significant.

RESULTS

Rate dependence of EADs in different models. To compare systematically the properties and determine the mechanisms of EADs under different conditions, we have established the following two EAD models using different pharmacological interventions: 1) isoproterenol (Iso; 100 nM) + BayK 8644 (BayK; 50 nM); and 2) H2O2 (200 μM).

We first assessed the PCL dependence of EADs in these two models. In the H2O2 model, EADs were consistently observed at a PCL of 6 s after the cell was exposed to 200 μM H2O2 for >6 min, while no EADs were observed when the PCL was shortened to 1 s (Fig. 1A). Conversely, EADs were apparently induced by Iso + BayK at a PCL of 1 s but disappeared at a PCL of 6 s (Fig. 1B). The incidence of EAD was examined by counting the number of EADs within 20 consecutive APs and the PCL dependence of EAD incidence in the different models plotted in Fig. 1C. It was clearly shown that the EAD incidence rate was higher at a long PCL (or slow rate) in the H2O2 model but at a short PCL (or fast rate) in the Iso + BayK model. These results suggest that H2O2-induced EADs are slow-rate dependent, consistent with our previous report (29), while Iso + BayK-induced EADs are fast-rate dependent.

Formation processes of EADs in different models. Different pacing rates may affect the level of [Ca2+]i. For example, pacing a myocyte at a rapid rate is thought to facilitate [Ca2+]i overload (7, 13). Thus, in the following experiments, we simultaneously recorded [Ca2+]i, transients (CaTs) and APs and compared the different behaviors of CaTs during EAD formation between the two models.

In the H2O2 model, EADs normally emerged 5–10 min after H2O2 (200 μM) perfusion. As shown in Fig. 2A, H2O2-induced spontaneous Ca transient (SCaT) occurred only when EADs were also observed in the corresponding APs. A significant increase of CaT amplitude was observed after the first EAD and SCaT occurred, suggesting sarcolemmal Ca2+ influx and SR Ca2+ content were enhanced during EADs, probably due to reactivation of ICa,L. Consistent with this notion, our and other’s previous studies (32, 42) have clearly demonstrated that H2O2 activates ICa,L, under either square pulse voltage clamp or AP waveform clamp.

In the Iso + BayK model, apparent changes in CaT and AP were caused with a very fast time course. In the case shown in Fig. 2B, both diastolic Ca2+ level and CaT amplitude gradually increased at ~10 s after Iso + BayK treatment. Particularly, apparent SCAts were observed from the sixth CaT with their amplitudes increasing progressively, correlating to the gradual prolongation of APDs at first, and then frank EADs (2B, bottom) when the amplitudes of corresponding SCAts were
higher. These results suggest Iso + BayK treatment leads to a progressive accumulation of \([Ca^{2+}]\), before EAD emergence. The emergence of CaWs/SCaTs preceded Iso + BayK-induced EADs, indicating CaWs were generated from spontaneous SR Ca\(^{2+}\) release rather than \(I_{Ca,L}\) reactivation. CaWs then caused \(I_{Ca,L}\), which contributed to EAD genesis in the Iso + BayK model.

Temporal relationship between EADs and corresponding SCaTs/CaWs. To further determine the primary cause (CaW vs. \(I_{Ca,L}\)) of EADs in each model, we next examined temporal correlation between EADs and SCaTs/CaWs by comparing the onsets of EAD upstrokes and the initiation of corresponding SCaTs/CaWs. It is conceivable that if an EAD is generated by a \(I_{Ca,L}\)-dominant mechanism, the corresponding SCaT will be the consequence of the EAD and therefore will occur subsequent to the EAD upstroke. On the other hand, if the EAD is generated by an SR-dependent mechanism, the spontaneous Ca release/CaW will be the cause of the EAD and would therefore precede the EAD upstroke. This rationale can be clearly discriminated, as illustrated by the results shown in Fig. 3.

In the H\(_2\)O\(_2\) model (Fig. 3A), the start of the EAD upstroke was significantly earlier than the initiation of the corresponding SCaT. The difference in initiation time (\(\Delta T\)) was 130 ms for this specific cell, and 113.5 ± 31.1 ms on average (means ± SD, \(n = 8\)). In the Iso + BayK model (Fig. 3B), however, the initiation of SCaT preceded corresponding EAD upstroke. The \(\Delta T\) was 50 ms for this specific cell, and 44.2 ± 8.7 ms on average (\(n = 9\)).

As previously mentioned, DADs were also induced in both models but with different time courses. While DADs or DAD-induced TAs always occurred in association with EADs in the Iso + BayK model (see Figs. 2–7), H\(_2\)O\(_2\)-induced DADs were only observed occasionally, required prolonged treatment with H\(_2\)O\(_2\), and arose much later than the emergence of EAD. In both the H\(_2\)O\(_2\) and Iso + BayK models (Fig. 3, C and D), it is apparent that the DADs were initiated after the beginning of corresponding CaWs/SCaTs, suggesting that Ca\(^{2+}\) overload-induced \(I_{NCX}\) is most likely to account for the DAD generation in both cases.

Distribution of EAD take-off potentials. Since some ionic currents (e.g., \(I_{Ca,L}\)) highly depend on membrane voltages, the take-off potentials (TOPs) at which the EADs occurred were also compared between these two models. In the H\(_2\)O\(_2\) model, EADs were consistently aroused at the end of phase 2 or early phase 3 of APs. The TOPs were within a narrow range (from +20 to −30 mV; Fig. 4A), which was consistent with that mediated by the window current of \(I_{Ca,L}\). (14).

On the other hand, the Iso + BayK-induced EADs occurred over a broader voltage range to a more negative level. As shown in Fig. 4B, the TOPs distributed over the whole AP repolarization phase (phases 2 and 3; from +20 to −60 mV). In addition, DADs and DAD-induced TAs (at phase 4) always appeared together in the same cell. These results suggest that in the Iso + BayK model, the charge carriers mediating EADs (as well as DADs) can be activated over a broad range of membrane potentials, in agreement with the voltage dependence of \(I_{NCX}\).

Relation between SCaTs/CaWs and EADs revealed by AP-clamp experiments. As pointed out previously, a SCaT/CaW might be either the consequence (i.e., sarcoplemmal-dependent mechanism) or the cause (i.e., SR-dependent mechanism) of an EAD. The following rationales were tested in a setting of AP-clamp experiments: If a SCaT is the consequence of an EAD, it should be suppressed by preferentially eliminating the EAD. Conversely, if a SCaT/CaW is the cause of an EAD, it should remain even if the EAD is preferentially eliminated.

In either model, EADs and SCaTs/CaWs were initially elicited in a cell under current-clamp mode. Thereafter, the myocyte was switched to voltage-clamp mode and the membrane potential was clamped under a normal AP waveform without EAD, which was recorded previously from a control myocyte. In the H\(_2\)O\(_2\) model, the SCaT was completely eliminated by switching current-clamp to AP-clamp mode with a

![Fig. 2. Formation process of EADs in different models. A: simultaneous recordings of whole cell (global) Ca\(^{2+}\) transients (CaTs) and APs at 5 min after treatment with 200 µM H\(_2\)O\(_2\). Note the increase of CaT amplitude (F/F\(_0\)) following the emergence of EADs. B: same as A, except that the myocyte was treated with Iso + BayK (10 s after treatment). Note the Ca\(^{2+}\) accumulation before the emergence of EADs. Spontaneous Ca\(^{2+}\) transients or Ca\(^{2+}\) waves (SCaTs/CaWs) are indicated by ○ and *, which correspond to APD prolongation and EADs (arrows), respectively. Spontaneous CaT (S) and triggered APs (T) are also shown.](http://ajpheart.physiology.org/doi/10.1152/ajpheart.00742.2011)
normal AP morphology (without EAD; Fig. 5A), indicating that the SCaT was caused by the corresponding EADs. In other words, the sarcolemma-dependent (or \( I_{Ca,L} \)-dominant) mechanism plays a predominant role in EAD genesis in \( H_2O_2 \) model.

In the Iso/H11001BayK model (Fig. 5B), however, the SCaTs/CaWs persisted even after the cell was switched to voltage-clamp under a normal AP without EADs, suggesting that SCaTs/CaWs occurred independently from membrane potential and seemed to be the primary cause for EAD formation under \([Ca^{2+}]_i\) overload condition. These results have provided the most convincing evidence on the cause-effect relationship between EADs and SCaTs/CaWs under different conditions.

**SR Ca\(^{2+}\) load and SCaT/CaW properties in the two EAD models.** To directly evaluate the SR Ca\(^{2+}\) content, the amplitudes of Ca transients induced by rapid exposure to 10 mM caffeine were measured. We observed SR Ca\(^{2+}\) was enhanced rapidly by Iso/H11001BayK, while there was no significant change in the \( H_2O_2 \) model (Table 1), suggesting an intracellular Ca\(^{2+}\) overload status in the Iso/H11001BayK model. Consistently, our results also revealed a significant elevation of CaT amplitude.
Fig. 5. Behaviors of SCaTs/CaWs under AP-clamp condition. After EADs were induced under current-clamp configuration (left), the recording from the same cell were switched to voltage-clamp configuration under an AP morphology without EAD (right) in H2O2 (A) and Iso + BayK (B) model. AP was recorded previously from a control cell. Note the SCaTs/CaWs were completely eliminated under AP-clamp condition in H2O2 model, while they persisted in Iso + BayK model.

Table 1. Comparison of Ca\(^{2+}\) handling properties in the two EAD models

<table>
<thead>
<tr>
<th>Model</th>
<th>CaT at Control</th>
<th>CaT After Treatment</th>
<th>Spontaneous CaT/CaW</th>
<th>SR Content at Control</th>
<th>SR Content After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O2</td>
<td>1.56 ± 0.27 (48)</td>
<td>1.54 ± 0.32 (42)</td>
<td>1.26 ± 0.09 (27)</td>
<td>1.67 ± 0.57 (19)</td>
<td>1.52 ± 0.35 (19)</td>
</tr>
<tr>
<td>Iso + BayK</td>
<td>1.44 ± 0.20 (44)</td>
<td>1.75 ± 0.37* (49)</td>
<td>1.65 ± 0.24 (32)</td>
<td>1.62 ± 0.35 (26)</td>
<td>1.99 ± 0.54 (24)</td>
</tr>
</tbody>
</table>

Values are means ± SD; numbers of calcium transients (CaTs) measured from multiple cells are indicated in the parenthesis. Amplitude (F/F0) of CaT, spontaneous CaT/CaW, and 10-mM caffeine-induced calcium release [sarcoplasmic reticulum (SR) content] are listed. EAD, early afterdepolarization; Iso, isoproterenol; BayK, BayK 8644. *P < 0.01, compared with CaT at control; †P < 0.01, compared with CaT after treatment; ‡P < 0.01, compared with SR content at control.

Effects of the I\text{NCX} inhibitor SEA0400. All above observations strongly suggest the contribution of I\text{Ca,L} vs. I\text{NCX} may vary (i.e., one of them is likely to play a predominant role) in EAD generation under different conditions. To determine directly the involvement of I\text{Ca,L} and I\text{NCX}, the effects of I\text{Ca,L} and I\text{NCX} inhibitors on the EADs and [Ca\(^{2+}\)], handling were evaluated in the two different EAD models.

SEA0400 has been used as a tool inhibitor of I\text{NCX} (5, 20). In the H2O2 model (Fig. 6A), SEA0400 not only abolished EADs, but also eliminated SCaTs, resembling the AP-clamp experiment shown in Fig. 5A. This result indicates that, although the primary current accounting for EAD formation is suggested to be the reactivation of I\text{Ca,L} in the H2O2 model, the inhibition of I\text{NCX} may reduce the inward current that is necessary for the generation of EADs.

In the Iso + BayK model, EADs were also efficiently abolished by SEA0400 (2 \(\mu\)M). However, SEA0400 exerted less effect on [Ca\(^{2+}\]), behavior such that the SCaTs/CaWs persisted (Fig. 6B), suggesting SEA0400 functioned at the downstream targets (i.e., NCX) of CaWs.

SEA0400 is the most potent Na\(^{+}\)/Ca\(^{2+}\) exchanger blocker available, although it is not completely specific and also inhibits L-type Ca\(^{2+}\) channels (5). While the level of SEA0400 we used (2 \(\mu\)M) may cause the maximal blockade of inward
Thus either increased inward currents or reduced outward currents, or both, promote EAD generation. For example, activation of inward late sodium current (I_{Na}), I_{Ca,L}, I_{NCX}, chloride current (I_{Cl}), as well as blockage of outward I_{Kr}, I_{Ks}, or I_{K1} have all been reported to mediate EAD genesis. Among these ionic mechanisms, CaW-mediated I_{NCX} (SR-dependent mechanism) and reactivation of I_{Ca,L} (sarcolemma-dependent mechanism) have been suggested as two major contributors for EAD genesis. However, their relative contributions underlying different pathological conditions are still under debate. In the present study, we conducted systematic comparison of two cellular models and focused on investigating the relative role of I_{Ca,L} vs. CaW-induced I_{NCX} in generating EADs by simultaneously recording APs and CaTs. We have provided important clues to identify different EAD mechanisms. For example, one significant result was that abnormal SCaTs accompanying EADs were abolished when the EADs were eliminated (by voltage clamp) in the H_{2}O_{2} model. However, this was not the case for Iso + BayK-induced SCaT/CaW and EADs, i.e., CaWs persisted even when EADs were eliminated (Fig. 5). Our results provide convincing evidence for the relative contributions of CaW-induced I_{NCX} and I_{Ca,L} in different EAD models. These two mechanisms are not mutually exclusive. They function coordinately to cause EADs; however, one mechanism may play a predominant role under a certain pathological condition.

**H_{2}O_{2} model**: I_{Ca,L}-predominant mechanism for EAD. ROS (including H_{2}O_{2}) have been suggested to mediate disturbances in the cardiac rhythm under various circumstances, such as heart failure, aging, and ischemia/reperfusion (22, 37). In the H_{2}O_{2} model: ICa,L-predominant mechanism for EAD. ROS (including H_{2}O_{2}) have been suggested to mediate disturbances in the cardiac rhythm under various circumstances, such as heart failure, aging, and ischemia/reperfusion (22, 37).
contrast to Iso + BayK, H$_2$O$_2$ seems to elicit EADs primarily by modulating membrane ionic channels. For example, we and others (32, 42) have shown that H$_2$O$_2$ (0.2–1 mM) increased both peak and plateau currents of I$_{Ca,L}$ and late I$_{Na}$ in rabbit ventricular myocytes. The activation of CaMKII, either directly via oxidative stress, or indirectly via elevated Cai,i is critical for these effects, which contribute to the arrhythmogenic potential of oxidative stress.

Consistent to classical EADs caused by sarcolemma-dependent mechanism, H$_2$O$_2$-induced EADs have been shown to be dependent on slow pacing rate (bradycardia). A previous computer modeling study (16) suggests that the delayed rectifier potassium current (I$_{Ks}$), which has a long activation time course and slow deactivation process, plays a key role in facilitating EAD generation at a slow pacing rate. In our most recent study (Zhao Z, Xie Y, Wen H, Xiao D, Allen C, Fefelova N, Dun W, Boyden PA, Qu Z, Xie LH, unpublished observations), we have demonstrated that I$_{Ks}$ is also an essential determinant for the slow rate dependence of EADs in rabbit ventricular myocytes due to its very slow recovery from inactivation. I$_{Ks}$, despite being a pure outward current, can potentiate EADs by lowering the AP plateau quickly into more negative voltages to allow I$_{Ca,L}$ reactivation before I$_{Ks}$ is fully activated to repolarize the myocyte, causing voltage oscillations in the plateau phase and thus EADs.

In addition to the difference in rate dependence, a difference in the TOPs of EAD upstrokes was also found between the two models. H$_2$O$_2$-induced EAD developed within a narrow voltage range (−30 to +20 mV), which is consistent with the I$_{Ca,L}$ window current. Other evidence such as CaT amplitude increase after EAD emergence, the temporal precedence of EAD upstrokes, and elimination of CaWs by clamping cell membrane potential with a normal AP (without a EAD) all support the primary role of I$_{Ca,L}$-dominant mechanism in EAD generation in H$_2$O$_2$ model.

I$_{NCX}$ functions predominantly in the forward mode, generating inward current during most of the AP repolarization. This is likely to lengthen the APD and form a “conditioning phase,” which facilitates reactivation of I$_{Ca,L}$ and/or I$_{Na}$ and generation of EAD upstroke (14, 40). It has been shown that various I$_{NCX}$ blockers (e.g., SEA0400, SN-6, and inhibitory peptide) shorten APDs under regular condition (1, 24, 35). Our present study showed that SEA0400 also eliminate H$_2$O$_2$-induced (I$_{Ca,L}$-dominant) EADs, as well as accompanying SCaTs (different from Iso + BayK model), which further support the “conditioning phase” mechanism. The SCaTs in the H$_2$O$_2$ model were most likely formed by Ca$^{2+}$ entry through reactivated I$_{Ca,L}$ and SR Ca$^{2+}$-induced Ca$^{2+}$ release triggered by the I$_{Ca,L}$ reactivation.

**Iso + BayK model: CaW-predominant mechanism for EAD.** It is well accepted that I$_{NCX}$ driven by CaW accounts for DAD formation (27, 34, 36). Recent experimental and simulation studies (13, 33) have demonstrated that CaW is also capable of evoking EADs. Pure β-adrenergic stimulation (Iso alone) has been used to cause EADs and DADs in dog myocytes (25, 39), and these could be facilitated by caffeine-enhanced SR Ca$^{2+}$ leak (23). In rabbit myocytes, however, Iso alone cannot efficiently induce EADs/DADs. This may suggest a high threshold for SR Ca$^{2+}$ spontaneous release in rabbit myocytes. In the present study, we were able consistently to generate frequent CaW and EADs/DADs by the application of Iso in the presence of BayK, as we demonstrated in our previous study (43). In this model, the SR Ca$^{2+}$ content may be increased by the activation of both I$_{Ca,L}$ and sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase activity. In addition, BayK has also been reported to increase RyR Ca$^{2+}$ leak (17), perhaps through a functional linkage between the sarcoplasmic dihydropyridine receptor and the SR ryanodine receptor (30). This may decrease the threshold for generation of CaW. In addition, the activation effect of BayK on I$_{Ca,L}$ (via changing both voltage dependence and single channel gating; Refs. 2, 28) may also lead to additional Ca$^{2+}$ overload. Therefore, spontaneous SR Ca$^{2+}$ release/CaW-induced elevation of cytosolic Ca$^{2+}$ may depolarize myocytes by the electrogenic I$_{NCX}$, which is most likely to primarily mediate EAD generation in Iso + BayK model.

Since intracellular Ca$^{2+}$ overload is exaggerated by fast pacing, EADs caused by spontaneous Ca$^{2+}$ release should be aggravated by fast pacing and tempered by slow pacing. This is proved to be true when we compared the EAD genesis at PCL of 6 vs. 1 s. It should be noted that Iso + BayK-induced EADs may be suppressed by further shortening PCL (i.e., from 800 to 300 ms), probably due to less I$_{Ca,L}$ recovery from inactivation when pacing rate is so fast.

Several other lines of evidence, such as CaT changes before or after EAD emergence, the temporal precedence of SCaT/DAD, less voltage dependence of EAD distribution, and suppression of EADs and DADs by inhibiting RyR with ryanodine, support the primary role of CaWs in EAD generation under Iso + BayK condition. The most convincing evidence came from the AP-clamp experiment (see Relation between SCaTs/CaWs and EADs revealed by AP-clamp experiments), in which the CaWs remained to appear even if the cell membrane APs were clamped without EADs. In addition, Ca handling (e.g., SR Ca$^{2+}$ content and SCaT amplitude) analysis provided direct evidence (see SR Ca$^{2+}$ load and SCaT/CaW properties in the two EAD models) for intracellular Ca$^{2+}$ overload in Iso + BayK model. Thus it is most likely that SCaT/CaWs-activated I$_{NCX}$ function as a primary underlying mechanism for both EAD and DAD formation in the Iso + BayK model, which may share a similar ionic mechanism with that of catecholaminergic polymorphic ventricular tachycardia (6, 10, 23).

**Synergy between I$_{Ca,L}$ and I$_{NCX}$ on EAD genesis.** Based on the above analysis, we suggest that the relative contributions of I$_{Ca,L}$ and I$_{NCX}$ may vary (i.e., one of them may play a primary role) in EAD generation under specific conditions. However, it is conceivable that the synergistic interactions between I$_{Ca,L}$ and I$_{NCX}$ are also present in the process of EAD generation [refer to a comprehensive review by Weiss et al. (41)]. For instance, reactivation of the I$_{Ca,L}$ during the plateau is likely to be the primary cause of EADs in H$_2$O$_2$ model. Meanwhile, enhanced window I$_{Ca,L}$ may trigger additional Ca$^{2+}$ release from the SR and increase I$_{NCX}$. Conversely, under the Ca overload condition (e.g., Iso + BayK model), spontaneous Ca$^{2+}$ release from the SR occurs to promote the forward mode of I$_{NCX}$, causing delay in repolarization, namely a conditional phase (44). This allows longer time for I$_{Ca,L}$ to recover from inactivation, which in turn favors EAD formation.

Since strong synergies are present between I$_{Ca,L}$ and I$_{NCX}$ in the generation of EADs, reducing either current may be effective in suppressing EADs. This has been proven to be true in
both the H$_2$O$_2$ and Iso + BayK models. Inhibition of ICa,L by nifedipine may suppress EADs in either model either by directly reducing the underlying inward Ca current or by secondarily attenuating the [Ca$^{2+}$]$_i$ overload and thus ICa,L. SEA0400, the most selective agent available to inhibit ICa,L, suppressed both EADs and SaCaTs in the H$_2$O$_2$ model, suggesting that ICa,L plays a facilitating role (conditional phase) in H$_2$O$_2$-induced EAD induction. The facilitated reactivation of ICa,L correlates to the ion carrier for both the formation of EAD and the trigger of SaCaTs in the H$_2$O$_2$ model. In the Iso + BayK model, however, SEA0400 inhibited EADs without affecting CaWs, suggesting a primary causal role of spontaneous SR Ca$^{2+}$ release on the EAD generation under the Ca$^{2+}$ overloaded condition. Several studies (11, 21) have shown that SEA0400 is effective to prevent/treat triggered activities and arrhythmias.

Relevance of EAD models. One may raise concerns that the EAD models we used in the present study might be too complex. We have tried to establish other “simple” models. Unfortunately, neither elevating extracellular Ca$^{2+}$ concentration (4–8 mM) nor Iso (up to 2 μM) alone successfully induced sustained EADs, although they might occasionally generate DADs. These results were consistent with previous reports showing that Iso alone was less efficient in causing sustained spontaneous Ca release and EADs/DADs (23, 38), since the reduction of SR threshold for spontaneous Ca$^{2+}$ release is also required (10). Therefore, we consider that the two EAD models used in the present study are the most ideal and stable approaches that enable us to make systematic comparisons for the relative contribution of ICa,L vs. CaW.

It should be noted that similar approaches have been utilized in the whole heart setting to induce EADs and triggered arrhythmias (22, 23), indicating the relevance of the models. In addition, it seems that the H$_2$O$_2$ model is more relevant to some pathological conditions. The H$_2$O$_2$ level in human blood plasma may reach as high as ~35 μM (12). It is also well known that ROS levels can increase under certain oxidative stressed conditions, such as chronic heart failure and ischemia-reperfusion (by as much as 100-fold; Ref. 8). Thus the concentrations (200 μM) used in most of our experiments are reasonably within its pathological range in situ.

H$_2$O$_2$-induced EADs were slow-rate dependent and were often observed at PCL >2 s. Accordingly, ROS-induced ventricular arrhythmias may be more readily implicated in the clinical setting of bradyarrhythmias (18), such as sinus-node dysfunction and atrial-ventricular conduction disturbances. It should be noted a heart rate at 10 beats/min is thought to be very deep bradyarrhythmia and mostly incompatible with life.

Limitation. One limitation is the complexity of the EAD models we used in the present study. While it is impossible to dissect one single current component that exclusively contributes to the generation of EAD, we have provided convincing evidence showing that either ICa,L or ISCX may play a primary role in one specific EAD model vs. another.

Conclusion. Our results have provided more convincing evidence for the heterogeneous mechanisms and their synergies for EAD generation. While EADs involve the complex interplay of several mechanisms, one mechanism (e.g., ICa,L reactivation vs. CaW-induced ISCX) may play a primary role under a certain pathological condition.

The classification (or nomenclature) of afterdepolarizations have been largely based on the timing they appear on the different phases during APD, i.e., EADs occur at phase 2 or 3 of an AP and DADs at phase 4 (after complete repolarization). Since afterdepolarizations (in particular EADs) may result from distinct cellular and ionic mechanisms, it may be more meaningful to further classify them based on their predominant mechanisms. As for the EADs induced in H$_2$O$_2$ and Iso + BayK models shown in the present study, we call them sarclemma-dependent and SR-dependent EADs, respectively.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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