Similarities between early and delayed afterdepolarizations induced by isoproterenol in canine ventricular myocytes

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Received 14 August 1996; accepted 22 November 1996

Abstract

Objectives: This study aims at clarifying the role of cellular Ca overload and spontaneous sarcoplasmic reticulum (SR) Ca release in the generation of early afterdepolarizations (EAD) by isoproterenol. The involvement of a Ca+-activated membrane current in isoproterenol-induced EAD is investigated. Methods: Membrane potential and contraction (an indicator of SR Ca release) were recorded in canine left ventricular myocytes at pacing cycle lengths (CL) of 300–400 ms. Threshold concentration for EAD was 20–50 nmol/l isoproterenol. Ni2+ (2.0–5.0 mmol/l) was used at normal and high (5.4 mmol/l) [Ca2+]i, to examine the role of Ca2+ current and/or Na+-Ca2+ exchange (\(I_{\text{Na-Ca}}\)) in EAD. Results: In all cells delayed afterdepolarizations (DAD) appeared during isoproterenol. In Na+-Ca2+ exchange current dominated (Ii) cells, EAD were also generated, which were fast-pacing dependent, occurring only at CL of 400–1000 ms. EAD were always initiated by a delay in repolarization. Early aftercontractions preceded the EAD upstrokes, often occurring without them. They coincided with the initial delays in repolarization. During treatment with isoproterenol, Ni2+ and high [Ca2+]i, EAD and DAD were suppressed despite the continued presence of early and delayed aftercontractions. Conclusions: Our data indicate that β-adrenergic EAD share a common ionic mechanism with DAD in terms of cellular Ca2+ overload and spontaneous SR Ca2+ release. β-Adrenergic EAD consist of two phases: (1) a conditional phase coinciding with the onset of an early aftercontraction, often followed by (2) an EAD upstroke. A Ca2+-activated membrane current, probably \(I_{\text{Na-Ca}}\), is necessary, at least for the initiation of these EAD.

Keywords: Early afterdepolarizations; Delayed afterdepolarizations; Isoproterenol; Na+/Ca2+ exchange; SR Ca2+ release; Excitation–contraction coupling; Dog, ventricular myocytes

1. Introduction

Both early (EAD) and delayed afterdepolarizations (DAD) have been incriminated in the generation of cardiac arrhythmias in patients [1].

DAD are generated under conditions that favor accumulation of cytoplasmic Ca2+ ([Ca2+]i), cellular Ca2+ overload and spontaneous Ca2+ release from the sarcoplasmic reticulum (SR). The DAD is caused by a Ca2+-activated inward current during spontaneous SR Ca2+ release: the Na+-Ca2+ exchange current \(I_{\text{Na-Ca}}\) [2,3] and/or non-selective cation current \(I_{\text{NS}}\) [4]. Together with DAD delayed aftercontractions are also generated and one may assume that spontaneously released Ca2+ ions from the SR activate the sarcolemmal inward current and the contractile system simultaneously [5].

EAD are afterdepolarizations that occur as transient retardations or reversals of the repolarization of the action potential [6]. Progress and agreement on their ionic mechanism(s) have been meager in comparison with DAD. This

Part of this work was presented in abstract form: Volders PGA, Szabo B, Kulcsar A, Lazzara R. Early aftercontractions precede early afterdepolarizations induced by isoproterenol in canine ventricular myocytes. PACE Pacing Clin Electrophysiol 1995;18:831(Abstract).
relates partly to the variety of conditions during which EAD are generated, indicating that there is not one type of EAD. In general, EAD are generated at slow pacing rates [drive cycle lengths (CL) longer than 1000 ms] and after prolongation of the repolarization. L- and T-type Ca$^{2+}$ current ($I_{CaL}$ and $I_{Cammu}$) as well as Na$^+$ current ($I_{Na}$) have been implicated as the currents underlying these EAD [1] (i.e., sarcolemma-dependent mechanisms).

β-Adrenergic receptor stimulation can induce both DAD and EAD. We [7,8] and other investigators [9,10] have suggested that cellular Ca$^{2+}$ overload and spontaneous SR Ca$^{2+}$ release are responsible for both afterdepolarizations in this condition via activation of a Ca$^{2+}$-dependent inward current (i.e., a SR-dependent mechanism).

The present study aims primarily at clarifying the role of cellular Ca$^{2+}$ overload and spontaneous SR Ca$^{2+}$ release in the generation of EAD by isoproterenol in canine left ventricular myocytes. It is hypothesized that if these EAD are generated by a SR-dependent mechanism, early aftercontractions are generated simultaneously. In contrast, if the EAD are generated by a sarcolemma-dependent mechanism, early aftercontractions will be present as a consequence of the EAD and will therefore occur after the EAD upstroke. In addition, block of the currents underlying the EAD will suppress early aftercontractions that are the consequence of the EAD (sarcolemma-dependent mechanism), but will not affect those that are the cause of EAD (SR-dependent mechanism).

2. Methods

This study was approved by the Animal Studies Subcommittee of the Department of Veterans Affairs, Oklahoma City, USA and the Committee for Experiments on Animals, University of Maastricht, Maastricht, The Netherlands, and conformed with the Guide for the care and use of laboratory animals published by the US National Institutes of Health.

2.1. Cell isolation procedure

The hearts of adult mongrel dogs anesthetized with pentobarbital (20–30 mg/kg i.v.) were quickly excised, washed, and a branch of the left anterior descending coronary artery was perfused with a cardioplegic solution at 10°C for 5 min. The perfusion was continued at 37°C with 100 ml of a nominally Ca$^{2+}$-free standard buffer solution and with collagenase at low [Ca$^{2+}$] (50 µmol/l) for 30 min. After washout with enzyme-free low-[Ca$^{2+}$] solution the tissue was perfused with the standard buffer solution containing 1.8 mmol/l [Ca$^{2+}$]. Finally, the tissue was minced into suspension, filtered and washed 5 times. Isolated myocytes were stored at 15°C temperature in either minimum essential medium or in standard buffer solution. No electrophysiological differences were observed between cells stored in either solution. The yield of rod-shaped myocytes from one dog heart was always between 60 and 80%.

2.2. Composition of solutions

The standard buffer solution contained (in mmol/l): NaCl 145, KCl 4.0, CaCl$_2$ 1.8, MgCl$_2$ 1.0, NaH$_2$PO$_4$ 1.0, glucose 11, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) 10, pH 7.4, with NaOH at 37°C and saturated with 100% O$_2$. KCl was set to 8.0 mmol/l in the cold cardioplegic solution. For the disaggregation process, collagenase (0.1% w/v Type II, Worthington Biochemical Co., Freehold, NJ or 0.08% w/v Type A, Boehringer Mannheim, Germany) was used to which was added bovine serum albumin (0.03% w/v). The storage medium used in part of the cell isolations was minimum essential medium enriched with Hanks’ salts (Gibco Laboratories #410-1600EA, Life Technologies, Inc., Grand Island, NY) and with (in mmol/l): NaHCO$_3$ 24, glucose 11, tauroine 10, pyruvic acid 2.0, ribose 5.0, allopurinol 0.1, pH 7.4, equilibrated with 5% CO$_2$ and 95% O$_2$ at 15°C. Isoproterenol was dissolved in distilled water containing 30 µmol/l ascorbic acid, kept at 5°C and in the dark. NiCl$_2$ (2.0–5.0 mmol/l) was added to the standard buffer solution with or without isoproterenol at a [Ca$^{2+}$] of 1.8 or 5.4 mmol/l for inhibition of $I_{Ca}$ and $I_{Na-Ca}$ (see Section 3). All of the chemicals used were of reagent grade and cell-culture-tested purchased from Sigma Chemical Co., St. Louis, MO. Almokalant was kindly provided by ASTRA (Göteborg, Sweden).

2.3. Electrophysiological and contraction recordings

A sample of the cell suspension was transferred to a water-jacketed perfusion chamber (0.5 ml) and superfused with standard buffer solution at a rate of 3 ml/min and at 37 ± 0.5°C. Cells were allowed to stabilize at CL of 1000 ms for at least 10 min before the start of the recordings. Myocytes used for the experiments were selected on the basis of the following criteria: having sharp striations, clear contours, transparent cytoplasms without granulations and blebs, and behaving electrophysiologically normal upon impalement. Cells could be used for experiments until 2 days after isolation without electrophysiological deterioration. In this period the arrhythmic response of myocytes to isoproterenol was invariably high and it was fully reversible upon washout of the agent.

Membranepotential recordings and intracellular stimulation were performed with standard glass microelectrodes (3.0 mol/l KCl, 30–60 MΩ) connected to a microelectrode amplifier (Axoclamp-2A, Axon Instruments, Inc., Foster City, CA). Myocyte contractions were imaged by a video camera (Panasonic GP-CD60, Panasonic Industrial Co., Secaucus, NJ) connected to an inverted microscope (Nikon, Inc., Melville, NY) and a video edge motion
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3. Results

3.1. The effect of isoproterenol on the ventricular action potential and contraction

In all myocytes a concentration of isoproterenol as low as 20 nmol/l consistently induced increased twitch contractions, followed by the appearance of DAD and delayed aftercontractions. For the generation of EAD, we used the same or slightly higher concentrations (up to 50 nmol/l), inducing EAD in approximately 70% of all cells tested.

2.4. Statistics

The data are expressed as means ± s.e.m. Statistical comparisons were made with the Student’s t-test and the paired t-test for unpaired and paired data groups, respectively, after testing for normality of distribution. Differences were considered significant if $P < 0.05$.
Fig. 3. Rate dependency of isoproterenol- vs. almokalant-induced EAD in canine left ventricular myocytes. Pacing CL of 300, 400, 500, 1000, 2000, 3000 and 4000 ms are applied in all cells. Isoproterenol is administered at 20, 40 or 50 nmol/l until EAD appear; n = 7 cells; almokalant at 3.0 μmol/l in 5 cells. The mean number of EAD \( \pm \) s.e.m. is expressed per 10 consecutive beats during steady-state pacing for at least 30 s. Almokalant induces EAD only at CL ≥ 2000, 3000 and 4000 ms; isoproterenol only at CL ≤ 400, 500 and 1000 ms. There is no overlap of the CL at which EAD are induced by these agents.

In 10 cells from 6 dogs treated with 20 nmol/l of isoproterenol and stimulated at a CL of 1000 ms, the dome amplitude of the action potential increased from 100 ± 3 to 111 ± 2 mV \( (n = 10; \; P < 0.05) \). No effect on the resting membrane potential was measured, which was on average \(-84 ± 3\) mV at control. Twitch-contraction amplitudes increased to 683 ± 157% of the control values. These observations are in line with earlier findings in single myocytes [13,14].

Fig. 1 illustrates that isoproterenol at low concentration (20 nmol/l) had divergent effects on the action-potential duration at 95% of repolarization (APD\(_{95}\)) of the 10 myocytes: the APD\(_{95}\) either decreased \((47 ± 14\) ms, from 274 to 227 ms; \(n = 5; \; P < 0.05\)) or increased \((13 ± 5\) ms, from 242 to 255 ms; \(n = 5; \; P < 0.05\)). Changes in the corresponding twitch-contraction durations tended to follow the changes in APD\(_{95}\), but were less pronounced. In all 5 myocytes initially responding to isoproterenol with an increased APD\(_{95}\), EAD (as well as DAD) were generated upon continued treatment with the agent (Fig. 2). We did not see EAD in the absence of DAD. In the 5 myocytes with a decreased APD\(_{95}\), only DAD were observed.

After an initial phase with increasing contraction amplitudes, further treatment with isoproterenol rapidly led to an arrhythmogenic response characterized by the induction of EAD, DAD and aftercontractions (as illustrated in Fig. 2). Subsequently, DAD-triggered action potentials could occur that could deteriorate into sustained triggered rhythms (data not shown). EAD observed in our experiments did not trigger action potentials nor resulted in sustained triggered rhythms, as was observed earlier [9].

The enhancement of contraction observed in the initial phase of isoproterenol treatment continued in the presence of afterdepolarizations and aftercontractions (Fig. 2; arrow). In all 10 cells larger twitch-contraction amplitudes were intermittently measured in this phase (Fig. 2; \(\uparrow\)) to a maximum of 776 ± 164% of control amplitudes. This difference between the maximal twitch-contraction amplitudes before vs. after the first appearance of afterdepolarizations and aftercontractions (683 ± 157 vs. 776 ± 164% of control, respectively) was significant \((n = 10; \; P < 0.05)\). Such large twitch-contraction amplitudes appeared as stimulated contractions (not as aftercontractions; Fig. 2).

Fig. 4. Representative tracings showing generation of EAD at fast pacing rate by isoproterenol and at slow pacing rate by almokalant. In the presence of isoproterenol (40 nmol/l; left panels) or almokalant (3.0 μmol/l; right panels) myocytes are stimulated at CL = 500 ms (upper panels) and CL = 4000 ms (lower panels). Note the generation of EAD at CL = 500 ms during isoproterenol and at CL = 4000 ms during almokalant, but not under opposite conditions. Isoproterenol-induced EAD are accompanied by early aftercontractions, almokalant-induced EAD are not. Left y-axes denote membrane potential (mV) in the 4 panels; right y-axes, cell shortening (μm); x-axes, time (ms). Upper traces denote contraction; lower traces, action potentials.
3.2. EAD and early aftercontractions induced by isoprote-
renol are fast-pacing dependent: comparison with almokalant

Isoproterenol-induced EAD were always accompanied
by early aftercontractions. Early aftercontractions could
occur without EAD upstrokes, but always before full
repolarization of the action potential. Their gradual appear-
ance after an initial response with increasing contractions
and simultaneously with the appearance of DAD suggested
a role for cellular Ca\textsuperscript{2+} overload. If so, these EAD and
early aftercontractions would be exaggerated by fast pac-
ing and tempered by slow pacing. We tested this in an
additional series of 7 myocytes from 4 dogs. Threshold
concentrations of isoproterenol used to induce EAD were
20 nmol/l (n = 2 cells), 40 nmol/l (n = 2) and 50 nmol/l
(n = 3). We compared this with the rate dependency of
EAD induced by the agent almokalant at 3.0 µmol/l in 5
cells from the same 4 dogs. Almokalant is known to
possess class III activity. At nano- to micromolar concen-
trations it is a specific blocker of the rapidly-activating
component of delayed-rectifier K\textsuperscript{+} current (\(I_{\text{K}}\)) [15], like
other pure \(I_{\text{K}}\) blockers such as dofetilide and E-4031 [16].
These agents delay repolarization and promote EAD induc-
tion at slow pacing rates. Pacing CL of 300, 400, 500,
1000, 2000, 3000, and 4000 ms were applied in all cells.

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Fig. 5. Temporal relationship between EAD and early aftercontraction. Contraction (upper trace), first derivative of the membrane potential (middle trace) and action potential (lower trace) are shown for a cycle with an EAD exhibiting a frank upstroke (A) and one without an EAD upstroke (B). In A, a delay in repolarization precedes the upstroke of the EAD, as evidenced by the reversal towards positive levels of \(dV/dt_{\text{up}}\) (arrow). This delay is initiated at 240 ms after the upstroke of the action potential and is followed by the EAD upstroke at 310 ms. The start of an early aftercontraction is at 245 ms, at the same moment as the initial delay in repolarization. In B, an early aftercontraction appears even without a frank EAD upstroke. The retardation of repolarization, as evidenced by the reversal of \(dV/dt_{\text{up}}\) (arrow), is initiated at 313 ms; the start of the early aftercontraction at 309 ms. Note the presence of late-coupled DAD (•) and corresponding aftercontractions in A and B. The first derivatives of the action-potential upstrokes are artificially truncated.
Table 1
The take-off membrane potential of EAD upstrokes during isoproterenol and almokalant in relation to pacing CL

<table>
<thead>
<tr>
<th>Pacing CL (ms)</th>
<th>Take-off membrane potential (mV)</th>
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<tbody>
<tr>
<td></td>
<td>Isoproterenol</td>
</tr>
<tr>
<td>400</td>
<td>$-27 \pm 4^*$</td>
</tr>
<tr>
<td>500</td>
<td>$-19 \pm 3$</td>
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<tr>
<td>1000</td>
<td>$-17 \pm 2$</td>
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Values are averages ± s.e.m.

$^*$ $P < 0.05$, CL = 400 vs. 1000 ms. Isoproterenol, $n = 7$ cells. Almokalant, $n = 5$ cells.

given either isoproterenol or almokalant. We calculated the number of EAD in 10 consecutive action potentials after $\geq 30$ s of steady-state pacing at a given CL.

In Fig. 3 it is shown that almokalant-induced EAD occurred only at CL $\geq 2000$ ms, with an incidence increasing to 6 EAD per 10 consecutive beats at CL = 4000 ms in these 5 cells. No early aftercontractions were observed with these EAD. The rate dependency of isoproterenol-induced EAD and aftercontractions was completely different. Here, only at CL of 400–1000 ms were EAD observed with a peak incidence of 3 EAD per 10 consecutive beats at CL = 500 ms ($n = 7$ cells; Fig. 3). Representative action-potential and contraction traces at CL = 500 and 4000 ms are shown in Fig. 4. The mutual dependence between APD$_{95}$ and EAD was different for almokalant and isoproterenol. In the case of almokalant, EAD were generated upon an already prolonged action potential. During isoproterenol the APD$_{95}$ was increased whenever EAD were (to be) present in consecutive beats, but decreased between such beats (Fig. 4, upper left panel).

In addition to a difference in rate dependency, there was also a difference in the average take-off membrane potentials of EAD upstrokes in the presence of isoproterenol vs. almokalant. It is shown in Table 1 that the upstrokes of isoproterenol-induced EAD developed at more negative levels and at broader range. These levels could vary from close to the resting membrane potential to sometimes slightly positive voltages. There was a significant difference between the average take-off potentials of EAD generated at CL = 400 vs. 1000 ms during isoproterenol (Table 1).

In summary, (1) isoproterenol-induced EAD are fast-pacing-dependent; (2) their charge carrier(s) can be activated over a broad range of membrane potentials; and (3) isoproterenol-induced EAD appear to be mechanistically different from almokalant-induced EAD.

3.3. Early aftercontractions precede EAD upstrokes

To determine the onset of EAD accurately (i.e., the time and voltage of the start of the delay in repolarization), we used the first derivative ($dV/dt_{rep}$) of the membrane potential [12]. This is shown in Fig. 5 for beats in which a delay in the repolarization was either followed (Fig. 5A) or not followed (Fig. 5B) by a frank EAD upstroke.

We examined the temporal relationship between EAD and early aftercontractions in 100 action potentials, as

Fig. 6. Bar graph showing temporal comparison between aftercontractions and afterdepolarizations induced by isoproterenol (20 nmol/l). (A) Onset of aftercontractions vs. onset of DAD that are chosen from the recordings in 10 ventricular myocytes. No significant difference in timing is found. (B) Onset of early aftercontractions vs. the initial delays in repolarization (change in $dV/dt_{rep}$) in action potentials with EAD, and vs. the upstrokes of these EAD. The early aftercontractions coincide with the initial delays, but come significantly earlier than the EAD upstrokes. The data are averages ± s.e.m. for 100 DAD, EAD and their aftercontractions.
illustrated in the example of Fig. 5. The arrows in Fig. 5A,B indicate the moment of reversal of dV/dt_{rep}, and the same point times on the action potential and contraction. A summary of this temporal comparison is shown in Fig. 6. The initial delay in repolarization occurred at 228 ± 22 ms after the upstroke of the action potential at a membrane potential of 1 ± 3 mV. The take-off of the upstroke of the EAD came after 271 ± 16 ms at −17 ± 2 mV. The start of the early aftercontraction came at 238 ± 17 ms. This was significantly earlier than the start of the upstroke of the EAD (271 ± 16 ms; P < 0.05), but different from the initial delay in repolarization (228 ± 22 ms; P = NS). Early aftercontractions occurred often in the absence of EAD upstrokes (Fig. 5B), but never without the initial delay in repolarization. Generally, their amplitude was larger in the presence than the absence of EAD upstrokes, as illustrated in Fig. 5A,B.

For comparison we evaluated the temporal linking between DAD and delayed aftercontractions measured in 100 action potentials. It was found that the onset of DAD coincided with the onset of delayed aftercontractions in the same way as was found for the start of the delay in repolarization and the onset of early aftercontractions during EAD (Fig. 6).

Thus, the time course of isoproterenol-induced EAD is characterized by two phases: (1) a conditional phase [17] (i.e., the initial delay in repolarization); (2) an EAD upstroke. The onset of the early aftercontraction coincides with the conditional phase of the EAD (i.e., significantly earlier than the EAD upstroke).

### 3.4. Generation of early and delayed aftercontractions during inhibition of EAD and DAD by Ni^{2+}

To clarify the relationship between isoproterenol-induced EAD and early aftercontractions we used the cation Ni^{2+}, which in the millimolar range is a non-specific inhibitor of the Na^+-Ca^{2+} exchanger [3,18] and a blocker of I_{CaL} and I_{Cag} [19]. We reasoned that if the ionic current responsible for the EAD also induces the early aftercontraction (i.e., if the aftercontraction was secondary to the EAD [20]), elimination of the EAD must also eliminate the aftercontraction. On the other hand, if the early aftercontraction is induced by an intracellular mechanism (i.e., spontaneous release of Ca^{2+} from the SR) which also induces the ionic current(s) that produce EAD as we hypothesize, the aftercontraction may occur even when the sarcoplasmic currents causing the EAD are blocked and the EAD eliminated. However, aftercontractions due to spontaneous SR Ca^{2+} release would not occur in the presence of a complete block of I_{CaL}, because the primary contraction due to Ca^{2+}-induced Ca^{2+} release would also be attenuated. Therefore, we took advantage of the fact that a rise in [Ca^{2+}]_i can partially overcome the block of I_{CaL} by Ni^{2+} because the blocking effect of Ni^{2+} results from competitive binding to the divalent cation-binding side of the channel [19].

In separate voltage-clamp experiments in 3 myocytes, we confirmed the relief of Ni^{2+}-induced inhibition of I_{CaL} by high [Ca^{2+}]_i (data not shown).

Ni^{2+} was applied in 5 myocytes from 3 dogs at a concentration of 2.0 or 5.0 mmol/l. A Generation of DAD, EAD and aftercontractions left panel. The subsequent addition of 2.0 mmol/l of [Ca^{2+}]_i to 5.4 mmol/l of (in the continued presence of isoproterenol and Ni^{2+}) partially restores this effect, as evidenced by the reappearance of twitch contractions and aftercontractions. Note the early aftercontraction without a companion EAD second from right panel and the delayed aftercontraction without a DAD second from right panel. Similar results were obtained in replicate experiments on 3 cells. (B) DAD and delayed aftercontraction (onset at 796 and 792 ms after the upstroke of the action potential, respectively) are generated (left panel). With the subsequent addition of 2.0 mmol/l of [Ca^{2+}]_i and 5.4 mmol/l [Ca^{2+}]_i (right panel), the DAD is inhibited, while the aftercontraction remains present starting at 701 ms. In panels A and B, Ca indicates Ca and Ni, Ni, oo Ni in the continued presence of isoproterenol and Ni^{2+} was applied in 5 myocytes from 3 dogs at a concentration of 2.0 or 5.0 mmol/l. As shown for the cell in Fig. 7A, following the induction of EAD and DAD by isoproterenol (left panel), Ni^{2+} at normal [Ca^{2+}]_i (1.8
mmol/l) greatly attenuated the action potential and abolished all twitch contractions, aftercontractions and afterdepolarizations (second from left panel). Thus, Ni$^{2+}$ acted as a Ca$^{2+}$-channel inhibitor, preventing both Ca$^{2+}$-induced Ca$^{2+}$ release and spontaneous Ca$^{2+}$ release from the SR not loaded with Ca$^{2+}$. To offset this effect of Ni$^{2+}$ at least partly, the $[\mathrm{Ca}^{2+}]_s$ was increased to 5.4 mmol/l. Under these conditions there was sufficient $I_{\text{Ca,L}}$ to induce a primary contraction and early and delayed aftercontractions occurred in the absence of EAD and DAD, respectively (Fig. 7A, right two panels). The spike-and-dome configuration was still attenuated at this $[\mathrm{Ni}^{2+}]_o$. We confirmed these observations in 3 additional myocytes.

In the other cell showing only DAD, 2.0 mmol/l $[\mathrm{Ni}^{2+}]_o$ and high $[\mathrm{Ca}^{2+}]_s$ attenuated DAD, but not delayed aftercontractions (Fig. 7B, right panel). The spike-and-dome configuration of the action potential remained at this $[\mathrm{Ni}^{2+}]_o$. The washout of Ni$^{2+}$ and high $[\mathrm{Ca}^{2+}]_s$ rapidly reversed their actions and afterdepolarizations returned within minutes. In these Ni$^{2+}$ experiments, early and delayed aftercontractions behaved clearly the same as before. The presence of isoproterenol was essential for their generation: without isoproterenol, no aftercontractions.

The step from 1.8 to 5.4 mmol/l $[\mathrm{Ca}^{2+}]_s$, during Ni$^{2+}$ and isoproterenol was also essential to generate early and delayed aftercontractions during inhibition of EAD and DAD. Such a step in the absence of Ni$^{2+}$ resulted in contracture of the cells within seconds and therefore could not be used as control.

In summary, (1) early and delayed aftercontractions can occur independently from EAD and DAD, indicating that spontaneous SR Ca$^{2+}$ release is the primary event; (2) a Ni$^{2+}$-sensitive inward current is necessary for both EAD and DAD.

4. Discussion

4.1. Changes in the APD$_{95}$ and contraction by isoproterenol

In this study we observed that the APD$_{95}$ of canine left ventricular myocytes either increased or decreased during treatment with 20 mmol/l isoproterenol. Divergent effects of β-adrenergic agonists on the APD$_{95}$ have been reported earlier to occur through direct or indirect modulation of many components that determine the net repolarizing current [21,22], but not at the same concentration of the agonist. This duality of isoproterenol responses was often found in myocytes from the same dog heart, which makes it unlikely that our cell isolation procedure per se was responsible for increases of APD$_{95}$ and EAD generation. In addition, these effects disappeared fully upon wash-out of isoproterenol.

We did not isolate the cells according to their transmural site of origin. It is known from the work of Antzelveitch et al. [23] that epicardial and endocardial myocytes respond differently to β-adrenergic receptor agonists—i.e., both show action-potential shortening but this is more pronounced in epicardium than endocardium. These investigators have also described a unique population of mid-myocardial (M) cells with pharmacological responsiveness different from epicardial and endocardial cells. A weaker $I_k$ contributes to a longer action potential in M-cells, especially at slow pacing rates [24]. Preliminary data from their laboratory show that isoproterenol at 20 mmol/l causes prolongation of the action potential in M-cells, but not in epicardial and endocardial myocytes [Dr. C. Antzelveitch, personal communication]. This prolongation is greater at more rapid pacing rates, consistent with the possibility that a Ca$^{2+}$-activated inward current counteracts the weaker outward current to delay repolarization. Isoproterenol was shown to produce even more prolongation and EAD generation in canine left ventricular M cells, but not in epicardial or endocardial cells, when the contribution of $I_k$ was further reduced by E-4031 [25]. Therefore, one possible explanation for the duality of isoproterenol responses in our study could be a heterogeneity of the cell population with M-cells having an APD$_{95}$ increase, and epicardial and endocardial myocytes having an APD$_{95}$ decrease.

During β-adrenergic receptor stimulation of ventricular myocytes, increasing twitch contraction amplitudes arose from a stable diastolic baseline, suggesting a beat-to-beat accumulation of Ca$^{2+}$ in the SR without an increment of diastolic $[\mathrm{Ca}^{2+}]_m$ (as shown earlier [26]) and attributed to enhanced uptake of Ca$^{2+}$ by the SR [27]. In contrast to the study of Capogrossi et al. [28], the amplitude of twitch contractions of our myocytes was not maximal at the time of the first manifestation of DAD and aftercontractions, as in all cells significantly larger amplitudes were intermittently measured during the arrhythmogenic phase of treatment. Our data may indicate that in canine ventricular myocytes the amount of Ca$^{2+}$ loading of the SR necessary for spontaneous Ca$^{2+}$ release is lower than that for maximal inotropy, in line with the computations by Luo and Rudy [29]. Species-related differences in intracellular Ca$^{2+}$ handling [30] may explain these different findings: in rat myocytes [28] the SR is known to work near its capacity for Ca$^{2+}$ sequestration.

4.2. Role of cellular Ca$^{2+}$ overload in isoproterenol-induced EAD

In the present study, EAD and early aftercontractions were generated just after the first appearance of DAD and delayed aftercontractions, often in conjunction with the same action potential. EAD were never observed without DAD. The occurrence of EAD and early aftercontractions was increased by rapid pacing, an experimental condition known to promote cellular Ca$^{2+}$ loading. While the ‘optimal’ CL for these EAD was around 500 ms, they disap-
Peared at slow pacing (CL ≥ 2000 ms) and as such behaved completely differently from the EAD induced by almkalant. These links between the appearance of EAD and DAD, and between EAD and fast pacing, strongly support our hypothesis that isoproterenol-induced EAD are related to cellular Ca2+ overload.

Earlier, we demonstrated the simultaneous generation of EAD and DAD in ventricular myocytes under different conditions of Ca2+ loading [31]. In a recent preliminary study [32], the development of EAD in ventricular cells and Purkinje fibers exposed to E-4031 was shown to be promoted by the acceleration of stimulation from initially slow rates, indicating that increased [Ca2+]cyt could also be involved in the generation of those EAD.

4.3. Role of spontaneous SR Ca2+ release in isoproterenol-induced EAD

In this study, we recorded contraction as a physiological marker of Ca2+-induced and spontaneous Ca2+ release from the SR at relatively high temporal resolution (16 ms). The onset of early aftercontractions coincided with the initial delays in repolarization during EAD, on average more than 30 ms before the EAD upstrokes. It is recognized that contraction can lag behind [Ca2+]cyt in a complex manner [33]. As but one example, Berk et al. [34] showed that during DAD in rat ventricular myocytes the Ca2+ aftertransient coincided with Iq and both preceded the aftercontraction by 60–90 ms [34]. If we apply such delay to our recordings, it implies that the underlying Ca2+ aftertransients occurred 60–90 ms earlier than the early aftercontractions and even more early than the EAD upstrokes.

It has been proposed that early aftercontractions are the result of Ca2+-induced Ca2+ release and that sarcolemmal ‘window’ Ical (underlying the EAD) triggers this Ca2+-induced Ca2+ release [20,35]. However, our observation of the temporal coincidence between the onset of early aftercontractions and the conditional phase of EAD (but not the EAD upstroke) suggests the opposite, namely that spontaneous SR Ca2+ release activates Iq countering the repolarizing currents. This hypothesis predicts that aftercontractions based on spontaneous SR Ca2+ release can occur without EAD.

Our experiments with Ni2+ show that EAD can be eliminated completely without suppression of the early aftercontractions, demonstrating conclusively that the aftercontractions cannot all be secondary to the currents that induce EAD. Under these conditions, the aftercontractions cannot be due to Ca2+-induced Ca2+ release from a surge of Ical, that simultaneously also generates the EAD, as hypothesized by others [20,29,35]. Since the aftercontractions must be due to Ca2+ release from the SR that is not induced by transsarcolemmal Ca2+ current, the Ca2+ release responsible for the aftercontractions must be by definition spontaneous. Spontaneous Ca2+ release has been accepted as the basis for DAD and delayed aftercontractions. However, the precise mechanisms of spontaneous Ca2+ release are unclear. Although our observations do not exclude the possibility that some EAD with take-off membrane potentials more positive than −35 mV are generated by reactivation of Ical, they establish that some aftercontractions and their associated EAD are independent of Ical. With respect to the specific identity of the current carrier for the EAD associated with spontaneous Ca2+ release, our experiments are not as conclusive because unknown effects of Ni2+ may exist on other currents which may be candidates for generating EAD. The Ni2+ experiments demonstrate that aftercontractions are primary and not secondary and thus support the inference of a Ca2+-activated inward current in EAD.

With the use of fluorescent Ca2+ indicators, Ca2+ aftertransients of relatively large amplitude have been observed during isoproterenol-induced EAD [10,35]. Yamada and Corr described these transients as multiple peaks of [Ca2+]cyt, following the action-potential upstroke [10]. The temporal relationship between EAD and Ca2+ aftertransients could not be determined in these experiments, due to the low time resolution (40 ms) of the techniques used [35]. Ca2+ aftertransients were inhibited by ryanodine, thus identifying the SR as the source of Ca2+ [10]. However, whether these transients were the result of Ca2+-induced Ca2+ release or spontaneous Ca2+ release from the SR could not be distinguished with the use of ryanodine.

The trigger for ‘spontaneous’ Ca2+ release during EAD and DAD is unknown. β-Adrenergic receptor stimulation has been shown to augment the removal of Ca2+ from the cytoplasm both into the extracellular space and into the SR. This suggests that the triggering of ‘spontaneous’ Ca2+ release may be linked to a high level of Ca2+ in the SR. Recently, Ca2+ sparks have been observed, which have been presented as the elementary phenomenon of Ca2+-induced Ca2+ release [36]. It has been demonstrated that the fractional release of Ca2+ from the SR is increased with an increase of the SR Ca2+ content [37]. Therefore, it could be possible that in the presence of isoproterenol Ca2+ sparks are augmented both in amplitude and in number and that they trigger a general SR Ca2+ release as the result of their spatial and temporal summation, which has been shown to occur in myocytes loaded with Ca2+ by high [Ca2+]cyt [38]. In addition, the spontaneous Ca2+ release inducing early aftercontractions could be triggered by Ca2+ being removed from the myofilament space after the twitch contraction and encountering a suprathreshold gradient of Ca2+ over the SR membrane.

4.4. Isoproterenol-induced EAD are initiated by INa-Ca

Based on the results of our study we conclude that cellular Ca2+ overload and spontaneous SR Ca2+ release are responsible for isoproterenol-induced EAD via activation of a Ca2+-dependent inward current. This current
appeared to be Ni\(^{2+}\)-sensitive. Ni\(^{2+}\) is a blocker of \(I_{\text{CaL}}\), \(I_{\text{CaT}}\) and \(I_{\text{Na-Ca}}\). It has already been discussed that \(I_{\text{CaL}}\) or \(I_{\text{CaT}}\) are unlikely to be involved in the initiation of EAD and DAD, leaving \(I_{\text{Na-Ca}}\) as a serious candidate.

A role for \(I_{\text{Na-Ca}}\) is also supported by our observation that EAD could be activated over a broad range of membrane potentials. Schouten et al. [39] demonstrated that \(I_{\text{Na-Ca}}\) is present throughout repolarization. They indicated that an increase in the Ca\(^{2+}\) transient would enhance inward \(I_{\text{Na-Ca}}\) during the action potential and delay repolarization, a point confirmed in the comprehensive mathematical model of the electrical activity of the ventricular myocyte by Luo and Rudy [29]. These mechanisms could explain why a modest action-potential prolongation was the initial response in myocytes ultimately generating EAD during isoproterenol.

At least one other candidate for the Ca\(^{2+}\)-activated inward current has to be considered, namely \(I_{\text{NS}}\) [4]. However, the presence of Ca\(^{2+}\)-activated non-selective cation channels has not been demonstrated in canine ventricular myocytes, while the contribution of \(I_{\text{NS}}\) to \(I_{\text{T Ca}}\) in other species is not consistent (e.g., [40]). Moreover, on the basis of experiments with the cation Sr\(^{2+}\). Niggli [41] found that Ni\(^{2+}\) inhibited \(I_{\text{T Ca}}\) generated by the Na\(^{+}\)-Ca\(^{2+}\) exchanger, whereas it did not block current through the Ca\(^{2+}\)-activated non-selective cation channel. Our observation that Ni\(^{2+}\) completely suppressed both DAD and EAD suggests that \(I_{\text{NS}}\) was not involved. It remains possible, however, that Ni\(^{2+}\) blocks some as-yet-unidentified Ca\(^{2+}\)-activated inward current that could contribute to EAD.

Thus, our present results indicate that \(I_{\text{Na-Ca}}\) plays an important role at least in the initiation of isoproterenol-induced EAD. The initial delay in repolarization characterizing the conditional phase of these EAD may set the stage for other inward currents to become activated. In this way, \(I_{\text{CaL}}\) could also be activated, not as the original cause of the EAD, but rather as the consequence of the depolarization initiated by \(I_{\text{Na-Ca}}\).

### 4.5. Possible clinical implications

Our results in isolated myocytes suggest that β-adrenergic receptor stimulation of the working myocardium could be arrhythmogenic by two distinct mechanisms. With intracellular Ca\(^{2+}\) overload, DAD appear that can induce triggered action potentials and sustained triggered rhythms. The EAD do not trigger new action potentials simply because at the membrane potential of their origin peak \(I_{\text{Na}}\) cannot be activated. However, the EAD could induce electrical heterogeneity by causing a variable prolongation of repolarization. Regional differences of repolarization (i.e., dispersion) favor re-entry [42,43].

The present study supports a role for β-adrenergic receptor stimulation in facilitating the onset of ventricular tachyarrhythmias on the basis of EAD generation and electrical heterogeneity in the idiopathic long QT syndrome [44,45]. In addition, the contraction abnormalities that accompany EAD generation could provide the cellular basis for the wall motion abnormalities of patients suffering from this syndrome [46]. The alleviation of both the electrical and mechanical abnormalities in patients with the long QT syndrome by Ca\(^{2+}\)-channel blocking agents suggests that abnormal cellular Ca\(^{2+}\) handling is the common denominator. Ca\(^{2+}\)-channel blockade would prevent or diminish the accumulation of intracellular Ca\(^{2+}\) and thus inhibit the generation of EAD and aftercontractions by preventing spontaneous Ca\(^{2+}\) release from the SR.

### 4.6. Conclusions

It is concluded that afterdepolarizations and aftercontractions induced by isoproterenol can occur during systole and diastole. Their simultaneous occurrence indicates a common underlying mechanism, likely spontaneous SR Ca\(^{2+}\) release. The observation that the onset of early aftercontractions precedes the upstroke of EAD also supports the role of spontaneous SR Ca\(^{2+}\) release and indicates the involvement of a Ca\(^{2+}\)-activated current, most likely \(I_{\text{Na-Ca}}\). The inhibitory effect of Ni\(^{2+}\) on both EAD and DAD, but not on their simultaneous aftercontractions, provides further support for the involvement of spontaneous Ca\(^{2+}\) release and \(I_{\text{Na-Ca}}\) in afterdepolarizations induced by β-adrenergic receptor stimulation.

### Acknowledgements

This study was supported by grant HL-35047 from the National Institutes of Health, by the Presbyterian Health Foundation, and by the Wynand M. Pon Foundation (Leusden, The Netherlands). This material is based upon work supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs. The study was also supported by a VA Merit Review Grant. The ‘Stichting VSB Fonds’ (Utrecht, The Netherlands) is greatly acknowledged for providing a stipend to P.G.A.V. The authors wish to thank Eva Szabo for her expert technical assistance, Benjamin J. Scherlag, PhD (University of Oklahoma Health Sciences Center) for helpful comments in his review of the manuscript, and Charles Antzelevitch, PhD (Masonic Medical Research Laboratory, Utica, NY) for providing information on transmural differences in isoproterenol responses.

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