

Mechanisms of calcium transient and action potential alternans in cardiac cells and tissues

William T. Clusin

Stanford University School of Medicine, Stanford University Hospital, Stanford; and Palo Alto Veterans Affairs Hospital, Palo Alto, California

Clusin WT. Mechanisms of calcium transient and action potential alternans in cardiac cells and tissues. *Am J Physiol Heart Circ Physiol* 294: H1–H10, 2008. First published October 19, 2007; doi:10.1152/ajpheart.00802.2007.—Alternation of cardiac action potential duration (APD) from beat to beat and concurrent alternation of the amplitude of the calcium transient are regarded as important arrhythmia mechanisms. These phenomena are causally interrelated and can be reliably evoked by an increase in beat frequency or by ischemia. The first part of this historical review deals with the physiology of APD alternans. Sections recounting the evolution of knowledge about calcium-activated ion currents and calcium transient alternans are interspersed among sections describing the growth of the so-called “restitution hypothesis,” which involves time-dependent recovery of potassium channels (including their passage through pre-open states) as a function of diastolic interval. Major developments are generally in chronological order, but it is necessary to move back and forth between the two theories to respect the overall time line, which runs from about 1965 to the present. The concluding two sections deal with the pathophysiology of calcium transient and APD alternans during ischemia, which may be the basis for out-of-hospital cardiac arrest during the initial stages of acute myocardial infarction.

sarcoplasmic reticulum; arrhythmias

KNOWLEDGE ABOUT THE ORIGINS of cardiac arrhythmias has been greatly increased by the development of fluorescent intracellular calcium indicators in the mid-1980s. Soon after these indicators were introduced, Lee et al. (34) used indo 1-AM to obtain the first recordings of cytosolic calcium transients from intact hearts (Fig. 1A). The main motivation for the use of these indicators in intact hearts was confirmation of pharmacological evidence (11) that a reversible increase in intracellular calcium concentration ($[Ca^{2+}]_i$) might occur in the first few minutes of ischemia, before cell necrosis. This was confirmed in saline-perfused rabbit hearts by Lee et al. in 1988 (33) and by others. An unplanned spin-off of these experiments was the discovery that, after 2–4 min of stop-flow ischemia, the calcium transient alternates from beat to beat (Fig. 1, B and C). Since mechanical alternans also occurred in these experiments and had been described decades earlier, the term “calcium transient alternans” was coined by Lee et al. to describe this result. Within the same year, a relationship between mechanical alternans and “intracellular calcium alternans” was reported in ferret papillary muscles, the surface cells of which were injected with the calcium-sensitive photoprotein aequorin (29).

Before the development of practical calcium indicators, mechanical alternans was shown to be associated with beat-to-beat fluctuation in action potential duration (APD). The latter phenomenon causes alternation in the T wave of the ECG (T-wave alternans), which is especially common in ischemic hearts and immediately precedes the onset of ventricular fibril-

lation (VF). This observation suggests that calcium transient alternans may play a causal role in the genesis of VF during ischemia (33, 40). More recently, it has become possible to detect microvolt T-wave alternans in patients as part of a special exercise test (46). A negative-microvolt T-wave alternans test has predictive value in identifying patients who are at low risk for sudden cardiac death (5).

Experimental studies of whether calcium transient alternans can cause sudden death during myocardial infarction have been guided by studies of less common arrhythmias in which the role of fluctuations in cytosolic calcium has been shown unequivocally (11, 18). These include arrhythmias of digitalis toxicity, which are due to calcium-dependent delayed depolarizing afterpotentials (26), and catecholaminergic polymorphic ventricular tachycardia, which is due to rare mutations in the calcium release channel (ryanodine receptor) and related proteins (35). Although these conditions account for a small proportion of arrhythmic deaths, they are easier to study than the arrhythmias of coronary artery disease and, therefore, could serve as a model for the more common causes of sudden death.

EARLY MATHEMATICAL DESCRIPTIONS OF APD ALTERNANS

The most important early study of the mechanism of APD alternans was an empirical study by Nolasco and Dahlen published in 1968 (39). They showed that, after abrupt shortening of the cycle length, the APD usually assumed the new (shorter) steady-state value within three beats or showed damped oscillations around the new steady-state value. However, there were exceptional cases in which the shortening of cycle length caused a perpetual fluctuation of APD between

Address for reprint requests and other correspondence: W. T. Clusin, H-2152, Stanford Univ. School of Medicine, Stanford, CA 94305 (e-mail: wclusin@stanford.edu).

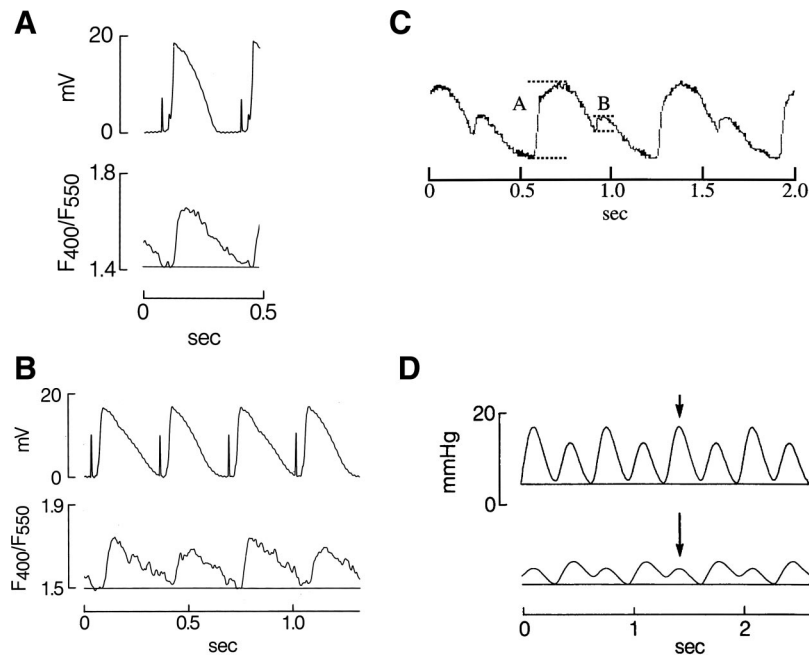


Fig. 1. *A*: recording of a monophasic action potential (MAP) obtained with the Franz MAP electrode from the epicardial surface of a saline-perfused rabbit heart (*top trace*) and a simultaneously recorded into 1 calcium transient obtained with an 11-mm fiber-optic probe (*bottom trace*). Calcium transient is ratio of fluorescence emissions at 400 nm (F_{400}), which increase with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) to fluorescence emissions at 550 nm (F_{550}), which decrease with a rise in $[Ca^{2+}]_i$. Heart is paced at 3 beats/s. [From Lee et al. (33).] *B*: recording similar to *A* from the surface of a heart that has been rendered ischemic for 2 min by cessation of flow of oxygenated saline. Action potential duration (APD) and $[Ca^{2+}]_i$ alternate from beat to beat. In calcium transient alternans, the taller calcium transient is preceded by a lower end-diastolic fluorescence level and accompanied by the longer APD. [From Lee et al. (33).] *C*: recording of calcium transient alternans obtained with a high-resolution digital camera. Heart was loaded with a long-wavelength calcium indicator, rhod 2-AM, and then perfused with a blood-saline mixture. Recording is from one 0.25-mm-diameter pixel. Systolic and diastolic alternans occur, as in *B*. An alternans ratio can be calculated as $1 - B/A$, where B is the net amplitude of the short transient (from end diastolic to systolic) and A is the net amplitude of the tall transient. Resulting ratio was 81%, which is larger than could be obtained with the 11-mm probe (cf. *B*). Excitation wavelength was 532 nm, and emissions were >600 nm. [From Qian et al. (43).] *D*: mechanical alternans in a saline-perfused heart at 3 min of ischemia. Contractions recorded by an intracavitary balloon (*top*) are displayed with local contractions recorded by an epicardial strain gauge (*bottom*). Pattern of out-of-phase alternans (arrows) is due to spatial heterogeneity of calcium transient alternans (see Fig. 3A). [From Lee et al. (33).]

two new values, both of which were shorter than the original APD. They also correctly noted that the diastolic interval (i.e., the time from 90% or 100% repolarization to the next upstroke) is a very sensitive predictor of the duration of the subsequent action potential. On the basis of this observation, a graphical method was developed that predicted which cycle length changes would produce the above-mentioned results (equilibration in 3 beats, damped oscillations, or sustained alternans) and the exact sequence of cycle lengths that would be observed (Fig. 2). This graphical method involved a plot of APD (on the y-axis) against diastolic interval (on the x-axis) and construction of a restitution curve, which can be obtained by maintained stimulation at each cycle length (dashed line in Fig. 2) or with single premature stimuli (S1S2 stimulation; solid line in Fig. 2).

The results of Nolasco and Dahlen (39) did not explain the mechanism of APD alternans but merely showed that the complex behavior of cycle length following a rate change could be predicted by supposing that APD is controlled by a process or processes that recover in a reproducible, time-dependent fashion during the diastolic interval. At the time of the work of Nolasco and Dahlen, early voltage-clamp studies had shown that repolarization of the action potential is due to a slowly activating, voltage-dependent potassium current (I_K), which also deactivates slowly, and that the inward calcium current undergoes inactivation from which it gradually recov-

ers during phase 4. (Calcium inactivation was not yet known to depend on cytosolic calcium.) These processes could explain APD alternans, and, in the case of concordant alternans (strong contraction with longer APD), the mechanical alternans could result from control of tension by APD, as occurs in the frog heart (39, 50). However, discordant alternans (weak contraction with longer APD) was not easily explained until the effects of $[Ca^{2+}]_i$ on ion channels were understood (20).

DISCOVERY OF CALCIUM-ACTIVATED ION CURRENTS: FIRST POTASSIUM, THEN SODIUM

In 1971, Spear and Moore (50) were the first to suggest, on the basis of simultaneous recordings of action potential and contraction alternans, that the duration of the cardiac action potential might be controlled by cytosolic calcium: "This (information) allows the possibility that the increase in intracellular free calcium may have an influence on the time course of the action potential." This suggestion was highly conjectural and prophetic, because calcium-activated ion currents had not been described in any excitable membrane at that time.

The concept that $[Ca^{2+}]_i$ can affect membrane potential by ion channel gating or by electrogenic sodium/calcium exchange was established in the late 1970s, before the development of calcium indicators that could be used in the heart. In

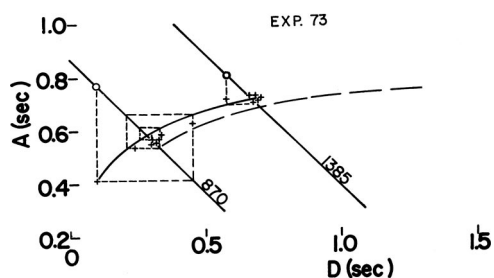


Fig. 2. Graphical method for predicting behavior of APD after a rate increase in frog ventricular strips. APD at 90% repolarization (A) is plotted vs. diastolic interval (D). Basic cycle length is 2,500 ms. Results are shown for abrupt shortening of the cycle length to 1,385 and 870 ms. Solid straight lines plot the following equation for the 2 new cycle lengths: cycle length = A + D. Curved dashed line and curved solid line show restitution curves obtained with steady-state and non-steady-state methods, respectively. \circ , 1st diastolic interval produced by the rate increase; +, observed APDs and diastolic intervals for the successive cycles. Graphical method consists of drawing a horizontal line to predict the next diastolic interval and then a vertical line to predict the next APD. This method predicts observed APDs with a high degree of accuracy. When cycle length is decreased to 870 ms, 4 cycles are required to reach the new steady state; at 1,385 ms, new steady state is reached in 2 cycles. [From Nolasco and Dahlen (39).]

July 1975, two studies in the nervous system [one involving a snail neuron (36) and one an electroreceptor (15)] showed that a component of the repolarizing potassium current is activated by the presence of calcium ions at the inner surface of the cell membrane. In the same year, Isenberg (23) reported that injection of calcium ions into cardiac Purkinje fibers caused a shortening of the action potential that varied with the amount of calcium injected. On the basis of this finding, Isenberg proposed that the slowly activating potassium current is regulated by $[Ca^{2+}]_i$ and that this might be the principal mode of gating. A similar suggestion was made by Bassingthwaite et al. (2), who used cyanide and zero sodium solutions to increase $[Ca^{2+}]_i$. However, the proposal that cardiac potassium currents are mainly controlled by calcium turned out to be incorrect.

The doctrine that repolarization in the heart is mostly due to slowly activating voltage-dependent potassium channels was rescued by the molecular cloning and characterization of the slowly and rapidly activating potassium channels, both of which are different from the calcium-activated potassium channels (large- and small-conductance calcium-activated potassium channels) in the nervous system. The rapidly activating potassium current (I_{Kr}) is thought to be purely voltage dependent, whereas the delayed rectifier potassium channel, I_{Ks} , passes more current at increased $[Ca^{2+}]_i$, according to the work of Tohse (51).

By the late 1970s and 1980s, it was appreciated that calcium ions play a role in the inactivation of the voltage-dependent calcium channel and can be cotransported across the membrane by a carrier (sodium/calcium exchange) with net movement of current. Because the electrogenic sodium/calcium exchange is more plentiful in the heart than in other tissues, an inward sodium current is the most common result of a rise in $[Ca^{2+}]_i$. Less common calcium-activated ion channels have also been discovered in the heart (Table 1) (11): 1) calcium-dependent chloride channels, which contribute to a varying extent (depending on cell type and species) to the initial repolarization of the action potential (phase 1); 2) calcium-activated nonselective

monovalent cation channels, which carry inward current at negative potentials and, therefore, have effects similar to electrogenic sodium/calcium exchange; and 3) small-conductance calcium-activated potassium channels, which are present in the nervous system and are also expressed in some cardiac tissues (57).

SPECIFIC EVIDENCE FOR A LINK BETWEEN CALCIUM TRANSIENT AND ACTION POTENTIAL ALTERNANS

In 1988, using the Franz monophasic action potential (MAP) electrode (Fig. 1B) in a saline-perfused rabbit heart, Lee et al. (33) obtained simultaneous recordings of calcium transient and action potential alternans. They postulated that calcium transient alternans might be responsible for the fluctuations in APD that produce T-wave alternans in a whole heart and that this could be a causal factor in the genesis of VF. Recordings with strain gauges showed that mechanical alternans could be out of phase at different points in a globally ischemic rabbit ventricle (Fig. 1D), and the idea of spatially discordant calcium and APD alternans producing VF during ischemia followed naturally. The inference of spatially discordant calcium transient alternans during ischemia was later confirmed in the same preparation by optical mapping (Fig. 3A) (43). At about the time the fluorescent calcium indicators were developed, it was found that the insect poison ryanodine blocks release of calcium from the sarcoplasmic reticulum (SR) and greatly attenuates the intracellular calcium transient. In 1989, Saitoh et al. (47) showed that ryanodine and high concentrations of caffeine can suppress APD alternans in canine papillary muscles impaled with microelectrodes.

Further advances in the study of calcium transient and APD alternans came with the discovery that these phenomena could be produced reliably in perfused hearts and single cells by a sudden and sufficient increase in the stimulation rate. That is to say, a nonischemic heart or isolated cell that contains a fluorescent calcium indicator and is paced at a rapid rate produces the same pattern of repetitive changes in the calcium transient and action potential that were described with ischemia (33). The ability to observe calcium transient alternans in single cells allows a number of fundamental observations to be made about the mechanism of alternans that could not otherwise be made (41). It is now known that APD and calcium transient alternans develop together when the stimulation rate is increased to a specific level, which is called the alternans threshold (40). To achieve such a rapid rate reliably, without loss of capture, the stimulus frequency must be increased in a series of steps. This

Table 1. Calcium-activated ion currents in the heart

Inward Current‡ (Prolongs APD)	Outward (or Less Inward) Current‡ (Shortens APD)
Na^+/Ca^{2+} exchanger†	L-type Ca^{2+} channel inactivation* Delayed rectifier K^+ channel (I_{Ks})*† Ca^{2+} -activated Cl^- channel*†
Nonselective Ca^{2+} -activated monovalent cation channel*†	Small-conductance Ca^{2+} -activated K^+ channel*†

APD, action potential duration. *Ion channel. † Ca^{2+} increases current and conductance. ‡Denotes effect of increased intracellular Ca^{2+} concentration.

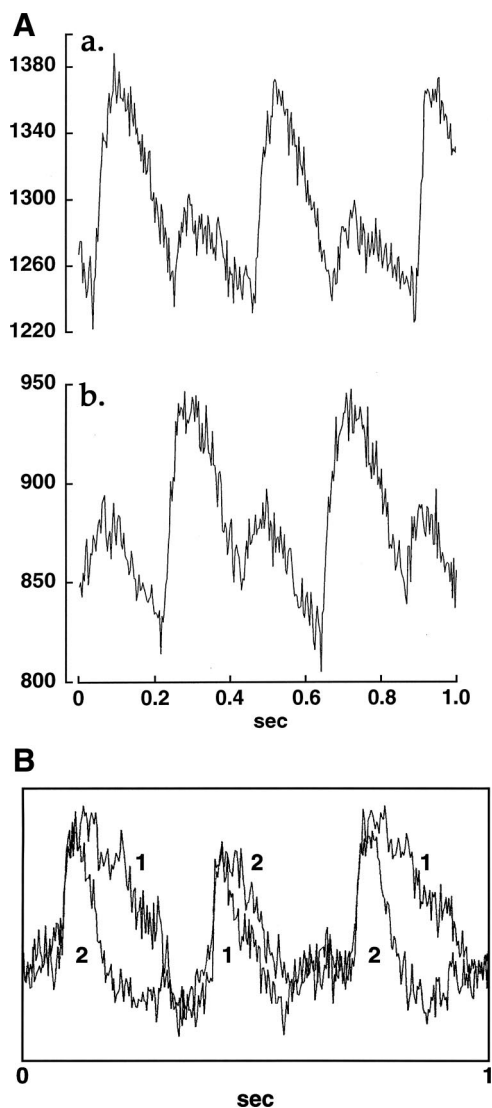


Fig. 3. A: calcium transients from 2 selected pixels in a rabbit heart loaded with rhod 2-AM, perfused with a blood-saline mixture, and then rendered ischemic. There is marked calcium transient alternans, which is out of phase in the 2 pixels, so that the first and odd transients are taller in *pixel a* and the second and even transients are taller in *pixel b*. Heart did not show alternans before ischemia and did not fibrillate. *Pixels a* and *b* are 10.6 mm apart and are part of a group of pixels that have alternans of the same phase on an alternans map. Excitation wavelength was 532 nm, and emissions were recorded at >600 nm (vertical scale). [From Qian et al. (43).] B: APD alternans in an experiment similar to A, where heart is stained with the potentiometric dye di-4-ANEPPS. Two superimposed recordings from the same heart show marked APD alternans that is out of phase. Degree of APD alternans is more marked than in the experiment with the MAP electrode (see Fig. 1C). *Pixels 1* and *2* are 4 mm apart. [From Qian et al. (44).] This degree of spatially discordant APD alternans could have caused ventricular fibrillation in an ischemic pig or dog heart.

is sometimes called “dynamic pacing” (25, 52).¹ Pruvot et al. (42) used multipixel imaging techniques to study the frequency dependence and spatial distribution of calcium transient and action potential alternans in guinea pig hearts coloaded with indo 1 and the potentiometric dye di-4-ANEPPS. They found

that APD and calcium alternans are spatially concordant (all regions have the same phase relationship) within a range of cycle lengths but become discordant (spatially out of phase) at extremely fast rates. In these experiments, onset of calcium transient and action potential alternans always occurred at the same time.

BRIEF REVIEW OF METHODS FOR STUDYING CALCIUM TRANSIENT ALTERNANS

The 20th anniversary of the first report of cytosolic calcium transients in an intact heart is 2007. Over these 20 years, approximately 100 papers have appeared in which fluorescent $[Ca^{2+}]_i$ indicators have been used in intact mammalian hearts. At least six fluorescent indicators have been used (8, 34, 56). Calcium transients were first recorded in whole hearts with fiber-optic probes, but more recent studies employ camera imaging techniques, in which thousands of pixels can be acquired in a few milliseconds, or confocal microscopy, where calcium transients can be observed in single cells within a perfused heart. Cytosolic calcium transients can be calibrated in single cells loaded with the free form of the indicator. Diastolic $[Ca^{2+}]_i$ is typically ~ 100 nM (0.1 μ M), whereas the systolic $[Ca^{2+}]_i$ is typically 1.0–1.3 μ M. Calibration can also be performed in intact hearts loaded with the cell-permeant (acetoxymethyl ester) form of the indicator, but this method is less precise. Fluorescent calcium indicators are quite sensitive to diastolic calcium. Decay of the calcium transient has been shown to follow an exponential time course (31), and the rate of decay can be correlated with the expression of the sarco-(endo)plasmic reticulum calcium-ATPase pump SERCA2a. When calcium transient alternans is recorded with fluorescent indicators, beat-to-beat alternation in the end-diastolic level of the transient is usually observed (Fig. 1, B and C). The fluctuation in diastolic level from beat to beat is more obvious in recordings obtained from single pixels (Fig. 1C). Fluctuations in diastolic calcium levels are usually not observed with aequorin.

A useful method for quantitation of calcium transient alternans is the alternans ratio devised by Wu and Clusin (56): $1 - B/A$, where B is the net amplitude of the short transient (from end diastolic to systolic) and A is the net amplitude of the tall transient (Fig. 1C). The alternans ratio has been used for a number of purposes, which include quantitative comparison of alternans under different experimental conditions (1, 56) and construction of alternans maps (43).

There has been particular effort in recent years to record calcium transients and action potentials simultaneously from large numbers of pixels in the same heart. One method involves coloaded the heart with the short-wavelength calcium indicator indo 1 and the potentiometric dye di-4-ANEPPS (32). With this method, two light sources, a UV and a visible-wavelength source, are used. A second method involves coloaded the heart with the long-wavelength indicator rhod 2 and an even longer-wavelength potentiometric dye, RH-237 (8). With this method, both indicators are excited by the same visible wavelength (~ 530 nm). Both of these methods have been used extensively and give similar results. However, when rhod 2 is used in the presence of the voltage dye RH-237, the resulting calcium transient is often distorted and acquires certain features of an action potential. This distortion may be due to time- and voltage-dependent changes in tissue transparency that occur when the heart is stained with RH-237. This effect probably does not

¹ Pacing of isolated cardiac myocytes at these rapid rates was not possible until myocytes that would tolerate rapid stimulation at 37°C could be made.

invalidate experimental results obtained with the combination of RH-237 and rhod 2.

MECHANISM OF ACTION POTENTIAL ALTERNANS: INSIGHTS GAINED FROM NEW TECHNIQUES

Potassium channel behavior. As noted above, APD alternans was originally explained by the fact that the diastolic interval determines the amount of residual potassium current at the onset of each action potential. In mammalian hearts, repolarization during phase 3 is largely due to the voltage-dependent potassium current, I_{Kr} , which activates with a time course of tens of milliseconds during an action potential (or depolarizing voltage step) and turns off with a similar time course when the membrane is fully repolarized. Rapidly and slowly activating potassium channels must pass through a series of pre-open states before they open, and they return to the resting state with some delay (49). Whenever a premature stimulus, or a decrease in cycle length, reduces the diastolic interval to near zero, the succeeding action potential is markedly shortened. This is due in part to cumulative or "residual" I_{Kr} and failure of the sodium and calcium current to recover fully from inactivation. (Residual activation of I_{Kr} may include channels left in a pre-open state.) I_{Ks} may also contribute to early repolarization following a short diastolic interval, because channels that reached a pre-open state during the first action potential are more likely to reach the open state during the second action potential (49).² If stimuli are now repeated at the new (shorter) cycle length, then the next action potential will be preceded by a longer diastolic interval, because the previous plateau was abbreviated. This action potential will therefore be of longer duration, but not as long as the action potentials at the original cycle length. During the third and subsequent action potentials, one of four patterns can be observed. 1) There may be no further change in APD after the second beat at the new cycle length (39). 2) There may be damped APD alternans, which converges to a new steady-state value (39, 52). 3) There may be sustained APD alternans (39, 52). 4) APD may continue to shorten until it asymptotically approaches its new steady-state value. This process has been termed APD accommodation (52).

There is no question that *patterns 1, 2, and 4* can be explained by the voltage-dependent gating of I_{Kr} and I_{Ks} . There have also been mathematical simulations in which *pattern 3* can be explained without calcium-activated ion currents in the model (54). However, the finding that complete elimination of the calcium transient prevents sustained alternans (19) suggests that the dynamic behavior of purely voltage-dependent ion currents is not a sufficient explanation. In addition, Lee et al. (33) showed that, during ischemia, calcium transient and action potential alternans could develop together, with no change in stimulus frequency and, therefore, no shortening or abrupt changes in diastolic interval.

Calcium transient alternans and calcium-activated currents. As noted above and in Table 1, a rise in $[Ca^{2+}]_i$ can activate a variety of ion channels or transporters, the relative prevalence

of which depends on the type of tissue and the species. Granted that calcium transient alternans is the cause of mechanical alternans, then it is certain that beat-to-beat fluctuations in ionic current must occur whenever there is mechanical alternans. The need to record calcium transients and action potentials from the same cell or from an electrically coupled region of tissue arises from the fact that calcium-activated ion currents could (in theory) produce either concordant calcium and APD alternans, in which the taller calcium transient accompanies the broader action potential, or discordant calcium and APD alternans, in which the taller calcium transient accompanies the briefer action potential. The ionic current changes shown in the left column of Table 1 would produce broader action potentials with the taller transients, whereas those in the right column would produce briefer action potentials with the taller transients. Since all cardiac cells have more than one calcium-activated or calcium-modulated current, the net effect will depend on which of these currents plays the predominant role. As noted earlier, the sodium/calcium exchange is especially prominent in all cardiac tissue, and the fact that larger calcium transients during alternans coincide with broader action potentials in several studies is consistent with mathematical models in which the inward sodium/calcium exchange current contributes to the maintenance of the plateau.

In order for calcium transient alternans to be considered the cause of accompanying APD alternans, it is necessary to show that, for a given cell within the intact heart, the two phenomena are inexorably linked. Although this had been claimed on the basis of recordings obtained with MAP electrodes (Fig. 1B), it was shown more rigorously by optical mapping studies (42). The fact that calcium transient and action potential alternans occur together is consistent with the hypothesis that the calcium transient "controls" APD but is not sufficient proof, because it is possible that purely voltage-dependent currents could produce APD alternans. In the frog heart, the amplitude of the calcium transient is strictly controlled by APD because of the lack of a well-developed SR. Although a longer action potential also favors net accumulation of calcium in mammalian cardiac cells, early experiments with the voltage-clamp technique suggested that modest fluctuations in APD from one beat to the next do not produce noticeable changes in the amount of calcium released by the SR (38).

A major step toward resolution of this problem was the experiment of Chudin et al. (9) shown in Fig. 4A. They produced action potential and calcium transient alternans in isolated rabbit cardiac myocytes by rapid pacing at physiological temperatures. In Fig. 4A, *left*, the action potential recorded by a patch pipette shows marked APD alternans, whereas the calcium transient shows calcium transient alternans. In this recording, the end-diastolic calcium level is lower before the taller calcium transients, which coincide with the longer action potentials (concordant alternans). In Fig. 4A, *right*, the cell is voltage clamped through the patch electrode (whole cell clamp) and a command pulse of fixed duration (equivalent to the longer action potential in the unclamped preparation) is used. Calcium transient alternans still occurs under these conditions, and its pattern is the same as that in Fig. 4A, *left*. This experiment shows that the development of calcium transient alternans during rapid pacing does not require APD alternans. The experiment also suggests that the calcium transients in the unclamped condition are not really being affected by the variations in APD, in contrast to the frog

² In mammalian hearts, the effect of I_{Ks} on APD depends on average heart rate. At slow rates, I_{Ks} has less effect. In humans, mutations of I_{Ks} or I_{Kr} can prolong APD and the QT interval, especially in homozygotes. However, total absence of I_{Kr} is very rare in humans and precludes normal gestation (21).

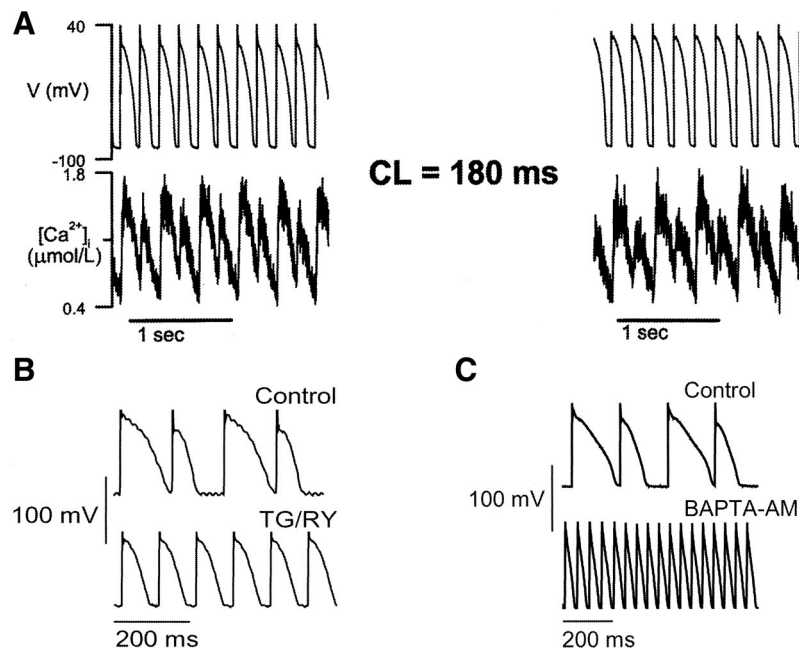


Fig. 4. A: APD and $[Ca^{2+}]_i$ transient alternans in an isolated rabbit ventricular myocyte during rapid pacing. *Left*: APD alternans is evident with no change in action potential amplitude. There is associated calcium transient alternans, with the tall $[Ca^{2+}]_i$ transient accompanying the broader action potentials. Diastolic $[Ca^{2+}]_i$ alternans also occurs. At *right*, a voltage-clamp circuit has been used to impose action potential waveforms of constant duration at the same frequency used for recording at *left*. Resulting calcium transients show the same degree of alternans as in the unclamped condition. [From Chudin et al. (9) with permission.] B: APD alternans in a rabbit ventricular myocyte paced at a cycle length (CL) of 133 ms recorded under control conditions (*top*) and in a myocyte that was pretreated with 10 μ M ryanodine (Ry) and 0.2 μ M thapsigargin (TG) to abolish the calcium transient (*bottom*). APD alternans could not be elicited under these conditions, even at a cycle length of 100 ms (600/min). [From Goldhaber et al. (19) with permission.] C: recordings obtained under control conditions similar to those described in B (*top*) and from a myocyte that was treated with 0.1 mM BAPTA-AM to abolish the calcium transient (*bottom*). Rapid pacing at 1,200/min fails to induce APD alternans. [From Goldhaber et al. (19) with permission.] A dynamic pacing protocol (progressively increasing rates) was used in A, B, and C. Effects of thapsigargin and BAPTA-AM on restitution (not shown) are shown in Ref. 19. Complex interplay between calcium cycling, restitution, and alternans is more fully explained in Ref. 19.

heart. The experiment of Chudin et al. has been repeated by others (53) and provides strong support for the hypothesis that calcium transient alternans is the cause of APD alternans.

Further evidence for a primary role of $[Ca^{2+}]_i$ in APD alternans was obtained by Goldhaber et al. (19), who treated isolated rabbit ventricular myocytes with thapsigargin and ryanodine, which was shown to abolish the calcium transient. Under these conditions, rapid stimulation failed to produce APD alternans, even though APD exhibited the normal shortening with decreasing cycle length (Fig. 4B). This experiment can be interpreted as showing that $[Ca^{2+}]_i$ plays an essential role in the genesis of APD alternans at high heart rates. Similar results were obtained when the cell-permeant calcium chelator BAPTA-AM was used to abolish the calcium transient. In cells treated with BAPTA-AM, stimulation rates as high as 1,200/min could be used without alternans or loss of capture (Fig. 4C). These experiments, together with the experiment of Chudin et al. (Fig. 4A) and the earlier work of Saitoh et al. (47), suggest that, for the conditions studied, calcium transient alternans is an important, and perhaps obligate, mechanism for the generation of APD alternans.

MECHANISM OF CALCIUM TRANSIENT ALTERNANS, INCLUDING SUBCELLULAR ALTERNANS

An obvious supposition about calcium transient alternans is that the amount of calcium stored in the SR might alternate, with larger stores before the large transient and smaller stores before the small transient. This is suggested by the occurrence

of diastolic calcium alternans, where removal of calcium is more complete (i.e., lower diastolic $[Ca^{2+}]_i$) before the tall transients. A related supposition is that the amount of calcium released from the SR must fluctuate from beat to beat, with less calcium released during the smaller transients. Two experimental approaches have been used to investigate these possibilities. Diaz et al. (16) used the method of rapidly superfusing voltage-clamped cardiac cells with caffeine (10) and taking the integrated sodium/calcium exchange current as a measure of the content of the SR. Caffeine pulses were timed to occur just before the onset of the taller transient or just before the onset of the smaller transient. (Experiments were done at room temperature with a special pulse protocol to allow observation of alternans at low stimulation rates.) These experiments indicate that the calcium content of the SR is higher before the larger transient. They also showed that perfusion of the cell with zero sodium just before the small transient could potentiate or restore the calcium transient, presumably by loading the SR with calcium.

Picht et al. (41) studied the mechanism of alternans by using a low-affinity calcium indicator (fluo-5N), which preferentially reports calcium in the lumen of the SR and registers a fluorescence decrease (depletion signal) proportional to the amount of calcium released. Diastolic fluo-5N fluorescence is presumably a measure of the filling of the stores just before calcium release. In some experiments, cytosolic calcium was measured concurrently using fura 2. In these experiments, rapid stimulation produced SR calcium depletion alternans, which stopped

when the stimulation frequency was reduced to below the alternans threshold (Fig. 5). Concurrent fura 2 recordings or recordings of cell edge movement showed that the larger calcium depletions coincided with the stronger contractions and larger cytosolic calcium transients. In some recordings, the fluo-5N signal showed diastolic fluctuations in which the SR was more loaded before larger depletions. However, there did not appear to be a monotonic or consistent relationship between the depletion amplitude and the preceding SR calcium level. Moreover, there were experiments in which depletion alternans occurred with no alternation in end-diastolic fluo-5N fluorescence. These observations were shown not to be due to saturation of the indicator. Picht et al. conclude that calcium transient alternans is due to fluctuation in the amount of calcium released from the SR from beat to beat. This is consistent with the conclusions of Diaz et al. (16) and is not disputed by any available evidence. However, in contrast to Diaz et al., Picht et al. conclude that a fluctuation in diastolic calcium content is not an obligatory or predominant cause of calcium transient alternans. They suggest that recovery of the ryanodine receptor calcium release channels might alternate from beat to beat, giving an alternating depletion signal (and calcium transient).

Previous work has shown that the probability of opening for the calcium release channel is higher when luminal calcium is increased (3). This mechanism may contribute to calcium transient alternans in situations similar to those shown in Fig. 5B, where diastolic SR calcium does alternate from beat to beat. However, the fact that depletion signal alternans can also occur without variation in end-diastolic SR calcium suggests that there is an additional explanation for alternate opening of ryanodine receptors during rapid stimulation.

Modern calcium imaging techniques, including confocal microscopy, have allowed calcium transient alternans to be observed in single cells and in specific parts of cells within an

intact heart. As noted above, calcium transient alternans in an intact heart can be out of phase (spatially discordant) during very rapid stimulation or during ischemia (Fig. 3A). In both situations, regions with alternans that is out of phase appear to be separated by a demarcation line on an alternans map. These maps are obtained from recordings in which hundreds or thousands of pixels, each containing many cardiac cells, are acquired simultaneously (40, 43). Aistrup et al. (1) used confocal microscopy to study the same problem at much higher spatial resolution in the rat heart. They found that, during onset of rapid pacing at a cycle length of 260 beats/min, a cell that showed no alternans could be only a few cells away from one with extreme alternans. When a plot of alternans ratio vs. beat frequency was obtained for cycle lengths near 260 ms, each cell had a characteristic cycle length at which alternans developed. Over this range of cycle lengths, alternans, when present, had the same phase relationship in every cell. However, at shorter cycle lengths (180–210 ms), alternans could be out of phase in neighboring cells, could occur in one part of a cell and not another, or could even be out of phase in two regions of the same cell. The latter two phenomena are referred to as subcellular alternans. Subcellular alternans has also been demonstrated in dissociated mammalian cardiac myocytes studied with confocal imaging (4, 22). Several mathematical treatments of calcium cycling dynamics have recently been published that could explain subcellular alternans (48, 55).

PATHOPHYSIOLOGY OF CALCIUM TRANSIENTS AND CALCIUM TRANSIENT ALTERNANS DURING ISCHEMIA

At about the time that calcium-activated ion currents were first discovered, pharmacological experiments suggested that impaired cellular calcium regulation might be the cause of early VF during cardiac ischemia (11). Pretreatment of animals with calcium channel blockers (11, 27) or β -adrenergic block-

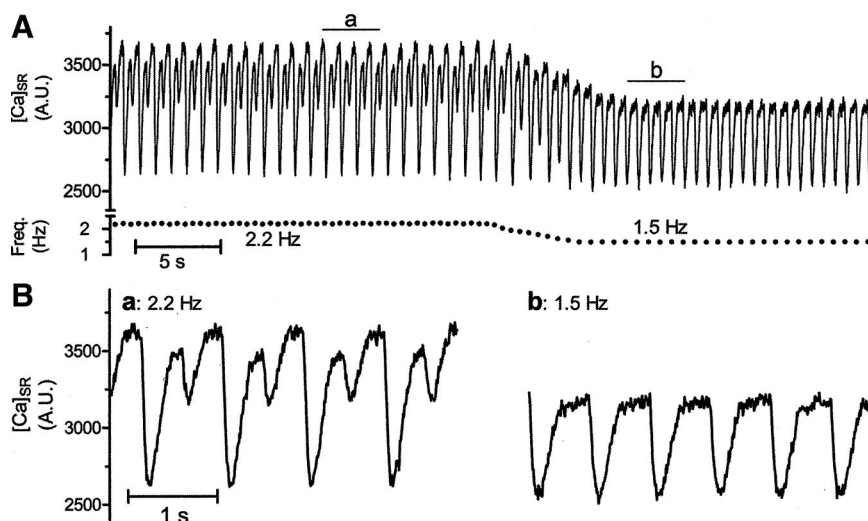


Fig. 5. Sarcoplasmic reticulum (SR) calcium transients recorded in a rabbit ventricular myocyte loaded with the membrane-permeable form of the fluorescent calcium indicator fluo-5N. High fluorescence levels reflect filling of the SR with calcium during diastole; brisk downward deflections denote release of calcium during systole. A shows the entire recording, and B shows short segments at expanded time scale. Stimulation frequency of 2.2 Hz (a) produces SR calcium depletion alternans. Larger depletions arise from a higher end-diastolic level. Reduction of the pacing frequency to 1.5 Hz (b) eliminates the alternans pattern and causes a fall in diastolic levels, indicating an overall reduction in cellular calcium stores. Separate recordings obtained at 2.0 Hz with the cytosolic indicator fura 2 showed systolic and diastolic calcium transient alternans similar to those in Fig. 1B. In some recordings, rapid stimulation produced alternans at the systolic level of the fluo-5N transient, but the diastolic level was constant. This finding was interpreted to indicate that availability of the ryanodine receptors to release calcium can fluctuate from beat to beat. AU, arbitrary units. [From Picht et al. (41) with permission.]

ers before coronary artery occlusion was shown to prevent VF or reduce the latency of onset in cases where VF is not prevented. Calcium channel blocker pretreatment slows the changes in the action potential that are produced by ischemia (33) and delays the appearance of ischemic "injury current" (14). The drug concentrations needed to produce these anti-ischemic effects are lower than those needed to flatten the restitution curve and prevent induction of APD alternans or VF by electrical stimulation (45). Pretreatment of perfused hearts with verapamil also prevents or slows the rise in $[Ca^{2+}]_i$ that normally occurs with ischemia (33). It has therefore been proposed (11) that pretreatment of an *in vivo* heart with calcium channel blockers causes parallel suppression of calcium transient alternans, APD alternans, mechanical alternans, and T-wave alternans during ischemia. However, the combined occurrence of all four effects remains to be shown, and any clinical benefit of this would be limited by the fact that, in contrast to β -adrenergic blockers, calcium channel blockers reduce the survival of patients with heart failure (11).

As noted above, there is less knowledge about the mechanism of calcium transient alternans during ischemia, because it is not possible to study ischemic cells in isolation with techniques such as voltage clamp or caffeine superfusion. Nevertheless, a number of observations have been made concerning the effects of ischemia on cytosolic calcium transients in blood- and saline-perfused hearts (33, 37, 43, 56). 1) Stop-flow ischemia does not cause immediate cessation of the calcium transients, even though the strength of contraction declines rapidly because of effects of acidity. 2) Ischemia causes a rise in the systolic and end-diastolic level of the calcium transient that is reversible on reperfusion. 3) Ischemia causes broadening of the calcium transient, which suggests a slowing of the process of calcium reuptake during ischemia. 4) Calcium transient alternans never develops during the 1st min of ischemia but, rather, typically develops between the 2nd and 4th min. During global stop-flow ischemia, there is a window of time during which calcium transient alternans is present and the pacemaker stimuli are still producing one-to-one capture. 5) Calcium transient alternans occurs to a greater degree in hearts that are perfused with a blood-saline mixture than in hearts perfused with saline alone (56). 6) Calcium transient alternans during ischemia can be out of phase in different regions of myocardium (Fig. 3A). This presumably leads to spatially discordant APD alternans (Fig. 3B). Spatially discordant APD alternans during ischemia has been reported to occur in the absence of beat-to-beat variations in conduction velocity (44). This is an important distinction from APD alternans produced by rapid pacing, where the development of spatially discordant APD alternans is thought to require conduction velocity restitution alternans (54).

Lakireddy et al. (30) obtained simultaneous recordings of calcium transients and action potentials in ischemic guinea pig hearts loaded with rhod 2 and RH-237. In the absence of ischemia, rapid pacing produced concordant alternans of the action potential and calcium transient. The cycle length at which calcium transient alternans was first observed was 212 ± 22 ms in the absence of ischemia and 550 ± 99 ms after 10 min of ischemia (cycle length during ischemia = 600 ms). In these experiments, 10 min of ischemia produced marked shortening of APD. These data agree with the earlier studies in the rabbit heart (33, 43, 56), where stop-flow ischemia pro-

duced calcium transient alternans at a maintained pacing cycle length of 333 ms. In the rabbit experiments, calcium transient alternans presumably occurred, because ischemia had caused the threshold cycle length for alternans to exceed the pacing cycle length.

Lakireddy et al. (30) also studied the ability of calcium transient alternans to produce APD alternans when cycle length was reduced from 600 to 300 ms after 10 min of ischemia. In some recordings, this produced APD alternans in which the longer action potential occurred with the taller calcium transient. This is also consistent with the observations in ischemic rabbit hearts. However, in other recordings, a similar degree of calcium transient alternans did not produce APD alternans. In the instances where APD alternans did not occur, the action potential before the rate jump was shorter than in cases where alternans occurred. This experiment confirms that calcium transient alternans is capable of producing concordant APD alternans (long action potential with tall transient) in an ischemic guinea pig heart but that in severe ischemia the ability of calcium transient alternans to produce APD alternans can be lost.

From the experiments that have been performed so far, it is possible to suggest specific molecular mechanisms for the development of calcium transient alternans during ischemia. Because calcium transient alternans does not occur until several minutes of ischemia, when the action potential is beginning to shorten, it can be concluded that cellular ATP levels must have declined, with a corresponding rise in ADP and inorganic phosphate. In addition, the supply of circulating fatty acids and glucose is cut off during ischemia. These changes can be expected to slow the operation of SERCA2a (28), which could explain the broadening of the calcium transient before the onset of alternans. Wan et al. (53) showed that the heart rate threshold for induction of calcium transient and APD alternans in the guinea pig heart is $\sim 25\%$ lower for the endocardium, which has a more slowly decaying calcium transient and less SERCA2a than the epicardium. If ischemia causes the initially nonischemic epicardial cells to behave more like normal endocardium because of reduced SERCA2a activity, then the appearance of calcium transient and APD alternans would be explained. The fact that heart rates that induce calcium transient alternans cause the SR calcium depletion signal to exhibit alternans is consistent with this formulation (41). However, the observation that calcium transient alternans can occur with no fluctuation in end-diastolic fluo-5N fluorescence suggests that ischemia may also affect the ryanodine receptors. Specific molecular mechanisms for calcium transient alternans involving the ryanodine receptors have been proposed by Blatter et al. (4) and others (22, 28) on the basis of the observation that perfusion of cat atrial myocytes with pyruvate (instead of glucose) produces calcium transient alternans.

Ischemia is a complex process involving rapid pH changes and free radical generation, in addition to ATP and substrate depletion. When intracellular pH is lowered from 7.3 to 6.5, the ryanodine receptor open probability is reduced by 50% (3). Acidity could therefore potentiate the metabolic effects proposed by Blatter et al. (4) and others (22, 28). Either of these factors could cause specific release channels to open only during even or odd beats. Calcium transient alternans and early ischemic cardiac arrhythmias are easier to demonstrate in blood-perfused hearts or *in vivo* than in a saline-perfused heart. Platelet release products (7) and thrombin (6) increase $[Ca^{2+}]_i$

in embryonic cardiac cell aggregates. It is therefore possible that extracellular messengers produced by normal blood and by a coronary thrombus contribute to development of calcium transient alternans and VF during ischemia.

CLINICAL IMPLICATIONS: FROM CELL TO CPR

It is estimated that 25% of the victims of acute myocardial infarction die in the prehospital phase. A substantial fraction of these deaths are due to the development of VF. VF during ischemia is much more likely to occur in a large animal (and, presumably, a human) than in a saline-perfused heart from a small animal, such as a rabbit, rat, or guinea pig. Experimental coronary ligation models have been used to identify drugs that might prevent sudden cardiac death if given on a daily basis to patients with risk factors. The most important class of drugs that have been shown to be beneficial in coronary ligation models and also prevent sudden death in humans are β -adrenergic blockers. β -Adrenergic blockers reduce calcium influx into cardiac cells and may, therefore, prevent the development of calcium transient alternans during experimental or spontaneous coronary occlusion (11).

The fact that T-wave alternans (which is indicative of APD alternans) precedes VF onset during ischemia suggests that calcium transient alternans is involved in the genesis of VF. An optical method that permits calcium transients to be recorded from the surface of an open-chest pig heart has been developed (12). However, these recordings are not stable enough to test the hypothesis that calcium transient alternans always develops before (or immediately before) VF onset. One must therefore extrapolate from small hearts to large hearts to determine whether the two phenomena develop under similar conditions.

When open-chest dogs are paced at 3 beats/s, widespread ischemia of the left ventricle (simultaneous occlusion of the left anterior descending and circumflex arteries) always causes VF, with a uniform temporal latency during serial occlusions in a group of dogs [mean VF latency = 138 ± 24 s, mean of 30 trials in 5 dogs (13)]. When nearly the same protocol (stop-flow ischemia at a heart rate of 3 beats/s) is used in a saline-perfused rabbit or guinea pig heart, calcium transient and action potential alternans are easily observed and also occur after 2–3 min of ischemia. Because calcium transient alternans develops under the same conditions in both species, these observations are consistent with the hypothesis that calcium transient alternans is necessary to cause ischemic VF.

An important question raised by these observations is whether combined calcium transient and APD alternans is always present somewhere in the ischemic zone at the onset of VF. Dilly and Lab (17) partly answered this question by recording MAPs from up to five sites in pigs during experimental coronary artery occlusion. They found APD alternans during ischemia in 16 of 16 pigs. APD alternans was present in 85% of electrodes in the ischemic zone, whereas none of the electrodes in truly normal myocardium showed APD alternans. All (100%) of the 163 episodes of APD alternans came from the ischemic zone or the border zone and none from the normally perfused myocardium.

An elegant illustration of the relationship between spatially discordant APD alternans and VF during ischemia was obtained by Janse et al. (24). Using four floating microelectrodes, they recorded action potentials from the left ventricle of a pig

heart and performed coronary ligation and, 4 min later, imposed an abrupt decrease in pacing cycle length from 400 to 300 ms. In all four recordings, the rate jump produced APD alternans that was out of phase during certain action potentials (cf. Fig. 3B). The APD alternans in the pig heart lasted only a few seconds, because the rhythm degenerated into VF. On the basis of the data of Lakireddy et al. (30), it can now be proposed that the decrease in cycle length from 400 to 300 ms caused the ischemic myocardium to abruptly surpass the rate threshold for calcium transient alternans. The above-described experiments suggest that, during coronary artery thrombosis in humans, the phenomenon of calcium transient alternans, with accompanying APD alternans, may be a common mechanism for the development of early VF and out-of-hospital cardiac arrest.

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