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ORIGINAL PAPER

Optical mapping study of blebbistatin-induced chaotic electrical activities in isolated rat atrium preparations

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Abstract We have studied the spatiotemporal pattern of blebbistatin-induced anomalous electrical activities in isolated rat atrial preparations using the optical mapping of excitation spread. Atrial preparations including the right or left auricle were dissected from adult rat hearts. Each preparation was then stained with a fast merocyanine–rhodanine voltage-sensitive dye (NK2761). Using a multi-element (16 × 16) photodiode array, we assessed the spread of excitation optically by timing the initiation of the action potential-related extrinsic absorption changes. The contraction-related optical signals were suppressed by adding (*S*)-(−)-blebbistatin (10–100 μM) to the bathing solution. Blebbistatin had an effective delay time of about 1.5 h following its application, at which time anomalous electrical activities occurred. These took the form of triggered activities and rhythmical spontaneous excitations. We optically mapped the spatiotemporal patterns of the excitation spread during these anomalous electrical activities. When the triggered activities occurred, the site of ectopic focus, where the triggered action potential first appeared, and the area of excitation spread varied in every event. When the rhythmical spontaneous excitations occurred, the excitation spread from the anomalous pacemaker and, occasionally, their spatial shift was observed. In addition, the combination pattern of the spontaneous excitations and triggered activities was also observed. We suggest that these phenomena are due to the disturbed intracellular calcium dynamics induced by the application of blebbistatin.

Keywords Anomalous excitation · Multiple-site optical recording · Rat atrium · (*S*)-(−)-blebbistatin · Voltage-sensitive dye

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Introduction

Multiple-site optical recording techniques have been used to monitor membrane potential in combination with a fast voltage-sensitive dye, which allows analysis of the spatiotemporal pattern of excitation spread [1, 2]. The results enabled optically mapping of the spread of excitation in isolated rat atrial preparations during an abnormal state of atrial rhythm evoked by electrical stimulation [3–6]. Excitatory waves occurred at a much faster rate during this abnormal state than in the normal condition, and the physiological pacemaker did not function. Optical mapping revealed that the re-entry mechanism was involved in this state.

During these studies, the suppression of optical signals due to muscle contraction (contraction-related optical signals, i.e., artifacts) was the most serious technical problem encountered in terms of obtaining fine optical action potentials. In order to reduce contraction-related optical signals, we used mechanical stretching as well as 2,3-butanedione monoxime (BDM: 20 mM) or cytochalasin D (Cyto D: 20–40 μM) [3–7]. These chemicals inhibit muscle contraction while having little effect on the electrical activities of the cardiac muscle. However, the inhibitory

effect of BDM is not complete, and the optical action potentials obtained were always contaminated to some degree with contraction-related signals. On the other hand, although the suppression of the contraction-related signals by Cyto D is much stronger than with BDM, we found that the former chemical has an effect on the conduction speed of the excitatory waves [7]. We have therefore been searching for a new chemical which would induce more effective suppression of the contraction-related signal without affecting the action potential and the excitation spread.

(*S*)-(−)-blebbistatin (Bleb) has recently been identified as a chemical that inhibits muscle contraction with a minimal effect on electrical excitation. Efimov and his group were the first to apply Bleb to Langendorff-perfused or isolated rabbit hearts and subsequently to isolated human A-V nodal preparations, with the aim of obtaining an optical recording of the cardiac action potential [8, 9].

Our first attempt to use Bleb was in isolated rat atrial preparations, where we evaluated its validity in the optical recording of action potentials. Throughout these experiments, we found that Bleb effectively suppressed the contraction-related optical signals without affecting the action potentials, indicating that Bleb is a good uncoupler of the excitation–contraction coupling for recording optical action potentials in isolated rat atrial preparations. However, in the course of these experiments, we encountered two unexpected anomalous electrical activities during the suppression of the contraction in the presence of Bleb. The first category of anomalous activities was triggered activity, which appeared following the action potential evoked by the electrical pacing. We termed this phenomenon "blebbistatin-induced triggered activity" (BIT). The second category was spontaneous rhythmical electrical excitation in the absence of extrinsic electrical stimulation, which we termed "blebbistatin-induced spontaneous electrical activity" (BIS). In the study reported here, we optically recorded these anomalous electrical activities and mapped the spatiotemporal patterns of the spread of excitatory waves with the aim of elucidating the characteristics of these two phenomena.

Materials and methods

Preparations

This study was approved by the Animal Care and Use Committee, University of the Ryukyus, and was conducted in accordance with its recommendations. Adult rats (Wistar strain, 200–500 g) of both sexes were anesthetized by inhalation of isoflurane. Each heart was quickly removed and bathed in an ice-cold bathing solution. The right or left auricle preparation was incised. The preparation was attached, positioning the endocardial side upward, to the silicone (KE106LTV; Shin-etsu Chemical, Tokyo, Japan) bottom of a simple chamber by pinning with tungsten wires, as described previously [3, 4, 6]. Preparations did not include the physiological pacemaker, and they did not exhibit spontaneous excitation. The preparations were kept in an oxygen-equilibrated bathing solution [(in mM): NaCl, 149; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.5; Tris HCl buffer, pH 7.4, 10; glucose, 10]. Bleb (10–100 μM) was added to the bathing solution to suppress optical artifacts due to contractile movements. The stock solution of Bleb was prepared as a 100 mM solution in dimethyl sulfoxide (DMSO).

Staining

The isolated atrial preparations were stained in bathing solution containing 0.5 mg/ml of a fast voltage-sensitive merocyanine–rhodanine dye (NK2761; Hayashibara Biochemical Laboratories Inc.) [10] for 30 min. The dye was first dissolved with small amount of DMSO for dispersion, followed by addition of the bathing solution. The final concentration of DMSO was 0.25% v/v. After staining, the preparations were washed with several changes of normal bathing solution. Neither phototoxic effects nor pharmacological actions could be observed using this dye, the bleaching time of which was fairly long [1–4, 6, 10].

Optical measurement

An optical recording system, designed to record the absorption change of NK2761 as an action potential-related optical signal, equipped with a 16 × 16-element photodiode array was used [4, 6]. In this system, the preparation chamber was mounted on the stage of a microscope (FLUOPHOTO-VFD; Nikon, Tokyo, Japan). Light from a 300 W tungsten–halogen lamp (JC-24 V/300 W; Kondo Sylvania, Tokyo, Japan) driven by a stable DC power supply (NL035-20; Takasago, Kawasaki, Japan) was collimated, rendered quasi-monochromatic with an interference filter with a transmission maximum at 701 ± 11 nm (Asahi Spectra, Tokyo, Japan), and focused on the preparation by means of a bright field condenser. The objective (×1) and the photographic eyepiece (×1) projected a real image of the preparation onto a 16 × 16-element silicon photodiode matrix array (C4675; Hamamatsu Photonics, Hamamatsu, Japan). Each pixel (element) of the array detected light transmitted by a square region (620 × 620 μm^2) of the preparation. The output of each detector in the diode array was fed into a PC-based recording system (ARGUS-50/PDA; Hamamatsu Photonics). The acquisition rate was 0.5, 1 or 2 ms/frame. Since the built-in software package in this recording system was

designed for neurophysiological experiments, several additional programs written originally by one of the authors (TS) were used for the display and the analysis of the data obtained from cardiac preparations. Using this optical recording system, we were able to simultaneously measure the cellular electrical activity from 256 contiguous areas in the preparation. The preparations were continuously paced by a bipolar electrode at the rate of 0.5 Hz (about threefold diastolic threshold intensity, duration 5 ms) except when the spontaneous electrical activities were being examined. All of the optical signals shown in this paper were obtained in a single sweep. Optical recordings were carried out in a still chamber without continuous perfusion of the bathing solution at a room temperature of 21–25°C. The incident light was turned off except during the measurement period in order to avoid bleaching of the dye.

Contraction-related artifacts could be easily discriminated from the action potential-related signal by their wavelength dependence; the action potential-related signals were completely eliminated at about 620 nm, the null wavelength of NK2761, whereas the contraction-related artifacts remained at 620 nm [1–4, 6]. (Due to a small mismatch between the true null wavelength of the dye and the peak wavelength of our "620 nm" interference filter, very small downward optical action potentials remained in the record at 620 nm in Fig. 1.)

Results

Suppression of contraction-related optical signals by Bleb

Figure 1 shows the inhibition of muscle contraction by Bleb. In this figure, optical signals recorded from two pixels (a and b) are represented. The leftmost two traces ("Control") are optical signals recorded in the normal solution. Although optical action potentials (indicated by arrowheads) could be recognized in trace a, the contraction-related signals (indicated by filled circles) were very large. In trace b, the optical action potential was covered with a huge contraction-related signal. In the traces obtained at 1 h after the application of Bleb (20 μM), the contraction-related signals became smaller, and optical action potentials could be observed quite clearly. Note that the vertical calibration is different between the leftmost traces and others. In the traces obtained at 2 h after the application of Bleb, the contraction-related signal became much smaller. In the traces recorded at 620 nm, the contraction-related signal was almost completely suppressed. (As stated in the Materials and methods section, very small optical action potentials remained in these traces.) As can

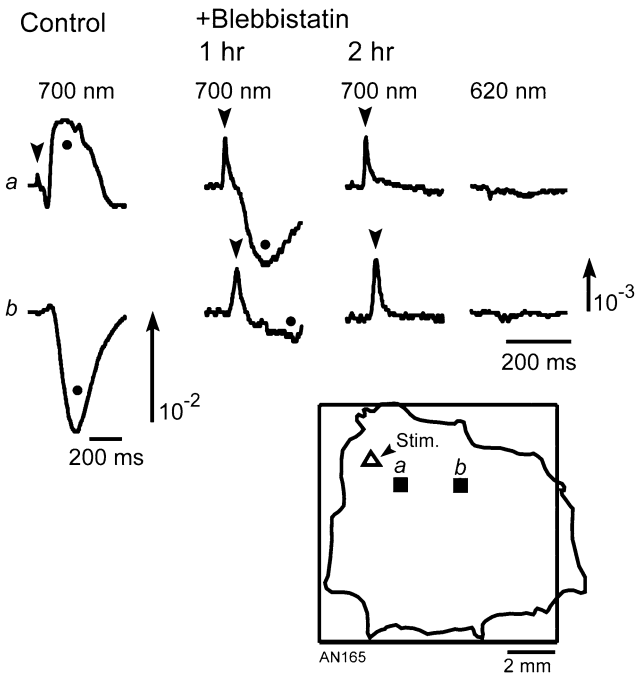

Fig. 1 Suppression of contraction-related signals by blebbistatin (Bleb). *a*, *b* Optical action potentials simultaneously detected by two elements of the photodiode array. The *leftmost* traces were recorded in a normal solution at 700 nm (*Control*). The *center* traces were recorded at 1 h after the application of Bleb (20 μM) at 700 nm. The *right two columns* of traces were recorded at 2 h after the application of Bleb at 700 and 620 nm, respectively. The *arrowheads* and *filled circles* indicate optical action potentials and contraction-related signals, respectively. In this and subsequent figures, the outputs of individual photodiodes have been divided by the resting light level (d.c.-background intensity = fractional change [ΔI/I]). The *direction of the arrows* on optical traces indicates a decrease in transmittance, and the *length of the arrows* represents the stated value of fractional change. Note that the vertical calibration is different between the leftmost traces and others. *Bottom right* Sketch of the preparation illustrates the location of the photodiode array on the preparation. *Large square* Position of the photodiode array, *filled squares indicated by a and b* positions of the pixel where the traces shown are recorded. The position of the stimulating electrode is indicated by an *open triangle labeled Stim*

be seen in this figure, the application of Bleb gradually inhibited the contraction of the isolated rat atrial preparation, but the action potentials could still be observed in the presence of Bleb.

The time course of contraction inhibition by the application of Bleb is shown in Fig. 2a. The data obtained from two different preparations at two different concentrations (open circles: 10 μM, filled circles: 100 μM) are shown. At both concentrations, the relative amplitudes of the contraction-related signals decreased exponentially after the application of Bleb, becoming almost completely suppressed at 2 h post-Bleb application. The inhibition of contraction was partially recovered after the removal of Bleb from the bathing solution (data not shown). In

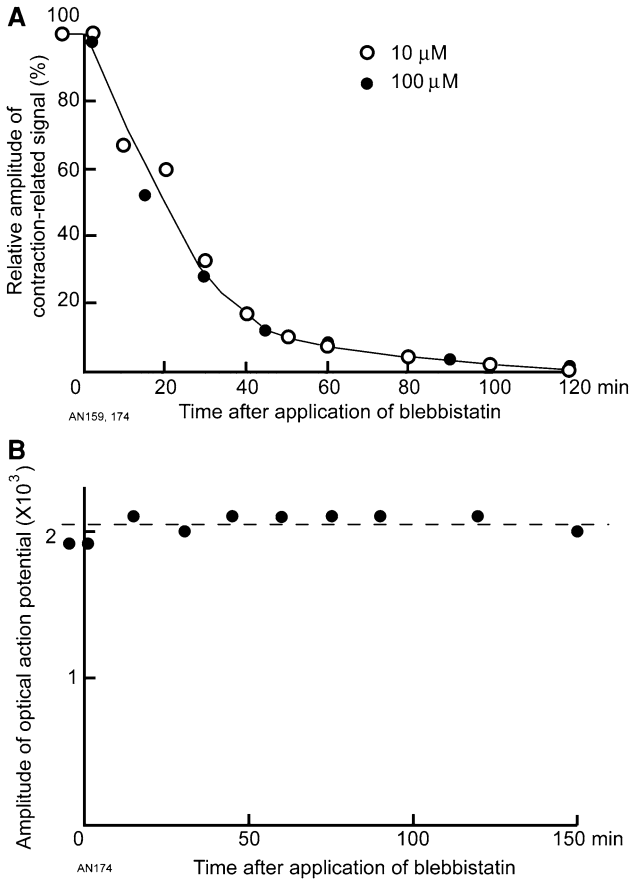

Fig. 2 The time course of the action of Bleb. **a** Time course of the suppression of contraction-related signals. The size of the normalized contraction-related signal (fractional changes in transmitted light) is plotted as a function of time after the application of Bleb. *Open circles* Data were obtained from a pixel of a preparation, and the concentration of Bleb was 10 μM. *Filled circles* Data were obtained from a pixel of another preparation, and the Bleb concentration was 100 μM. **b** The time course of the amplitude of the optical action potential. The concentration of Bleb was 100 μM. The *dotted line* indicates the mean value of the amplitude

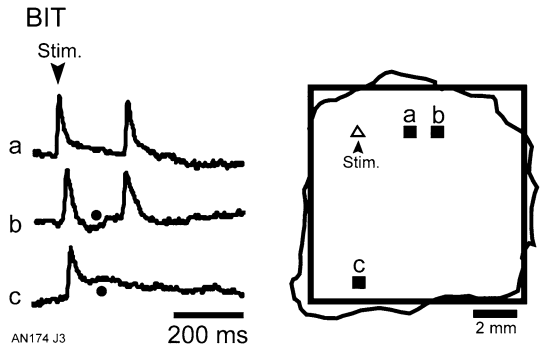

Fig. 3 Optical signals of the Bleb-induced triggered activity (*BIT*). *a*, *b*, *c* Optical action potentials simultaneously detected by three elements of the photodiode array. The traces were recorded at 700 nm 2 h after the application of Bleb (100 μM). *Right* Sketch of the preparation illustrates the location of the photodiode array on the preparation. Other symbols are as defined in Fig. 1

contrast, as shown in Fig. 2b, the amplitude of the optical action potentials remained constant in the presence of Bleb at 100 μM for 2.5 h. These results indicate that the application of Bleb suppresses the muscular contractions without affecting the action potentials in the isolated rat atrial preparations. Then, once the contraction-related optical signals were largely suppressed (i.e., 1.5–2 h after application of Bleb), BIT or BIS (or both) became observable.

Blebbistatin-induced triggered activity

In Figure 3, an example event of BIT is shown. This event of BIT occurred about 2 h after the application of Bleb (100 μM). In this figure, action potential-related optical signals obtained from three pixels (a–c) among the 256 pixels recorded simultaneously from the isolated atrial preparation using the 16 × 16-element photodiode array are shown. A sketch of the preparation imaged on the photodiode array and the positions of the individual elements in the matrix array are shown on the right. In traces a and b, optical action potentials, which were evoked by the electrical stimulation (indicated by "Stim."), were followed by other action potentials. In contrast, in trace c, only one action potential was observed, indicating that the area where BIT appeared was limited. These are typical records of BIT. We mapped the spatiotemporal pattern of the spread of optical action potentials during the events of BIT.

The maps of excitation spread during events of BIT are shown in Fig. 4. In these maps, the positions of the wavefront are displayed as isochrone curves with intervals of 20 ms. These maps were obtained from the same preparation as that shown in Fig. 3. The maps of excitation spread during the event of BIT shown in Fig. 3 are represented in Fig. 4a, b. Figure 4a shows the pattern of the excitation spread of the action potential evoked by an electrical stimulation. The excitation first appeared at the site of stimulation (the open triangle on the map indicated by "Stim") and spread radially toward all the parts of the preparation. The map of the excitation spread of the second action potential is shown in Fig. 4b. In this map, the excitation first appeared at an anomalous focus near the center of the recording area (the open circle indicated by "F") and spread toward only a restricted area of the preparation: the second action potentials were observed only from this restricted area of the preparation. Note that, in this map, the distances between the isochrone curves are narrower than those of the first (evoked) action potential (shown in Fig. 4a): the conduction velocity of the excitation spread was slower.

Figure 4c, d represents the maps of the second action potentials recorded in two other events of BIT in the same preparation. As shown in these maps and Fig. 4b, event-

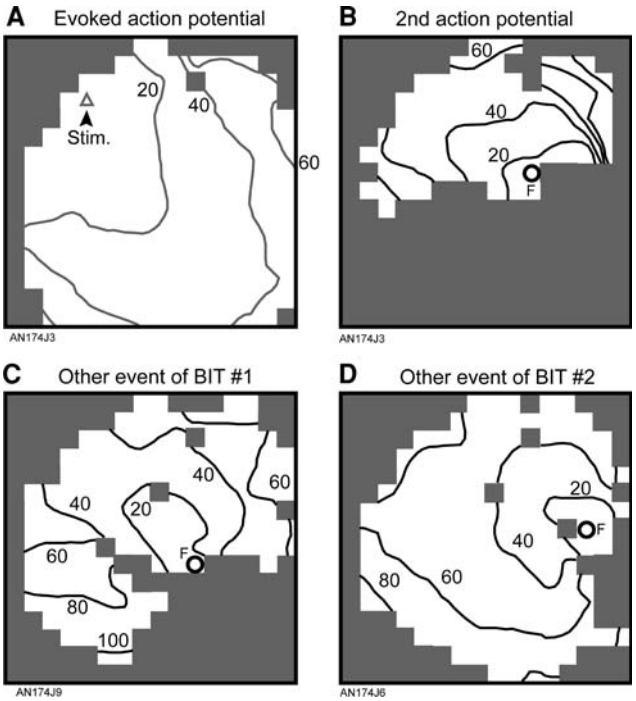

Fig. 4 Optical mapping of the BIT. **a** The map of the excitation spread for the evoked (first) optical action potentials (shown in Fig. 3). *Open triangle with Stim* Position of the stimulation. **b** Map of the excitation spread for the second optical action potentials (shown in Fig. 3). *Open circle with F* Position of the anomalous focus where the second action potential first appeared. **c**, **d** Maps of the excitation spread for the second optical action potential of two other events of BIT. The positions of the wavefronts are displayed as *isochrone curves* at 20-ms intervals. *Peripheral gray areas* Areas where the delay could not be measured due to the absence of the signals or the preparation

to-event variations in the spatiotemporal patterns of the excitation spread were observed among the events of BIT. In Fig. 4c, the excitation first appeared at an anomalous focus near the center of the recording area, subsequently spreading toward a restricted area of the preparation (but not the same area as in Fig. 4b). In Fig. 4d, the excitation first appeared at an anomalous focus on the right side of the recording area, subsequently spreading to a wider area than those shown in Fig. 4b and c. It should be noted that the triggered activities appeared with their own anomalous foci and that not only the positions of the foci but also the areas of excitation spread varied among the events of BIT.

Figure 5 shows an event of BIT which exhibited a unique pattern of excitation spread; it was observed 2 h after the application of Bleb (10 μM). Optical signals simultaneously recorded by three pixels are displayed in Fig. 5a. Three optical action potentials are recorded in trace b, whereas two action potentials are recorded in the other traces. A sketch of the preparation imaged on the photodiode array and the positions of the individual pixels in the matrix array are shown on the right of the figure. In Fig. 5b, the maps of the excitation spread of the second action potentials are shown. In these maps, the intervals of isochrone curves are 50 ms. The timing of the appearance of the second action potential at the anomalous focus in the center of the recording area (open circle indicated by "F" in the left map) is regarded as time 0 ms (about 200 ms after the stimulation). As can be seen in the left map (0–200 ms), the excitatory waves spread upward and downward from the anomalous focus. Then, in the right map (200–300 ms), the excitatory waves converged toward the central area again and were interrupted by the linear functional blocked area over which excitation cannot conduct (bold line indicated by "B"). This unique spread pattern of the excitation resulted in the three optical action potentials in the central area and was observed only once in this preparation in all experiments.

Blebbistatin-induced spontaneous electrical activity

The other type of anomalous electrical activity induced by Bleb is BIS. Figure 6a is an example of the optical recording of BIS that was observed 2 h after the application of Bleb (50 μM). In this figure, simultaneously recorded action potential-related optical signals obtained from four pixels (p and a–c) are shown. A sketch of the preparation imaged on the photodiode array and the positions of the individual elements in the matrix array are shown on the bottom right. Spontaneous optical action potentials appeared rhythmically. The occurrence of the events of BIS was identified by the rhythmical appearance of spontaneous optical action potentials after the pacing stimulations were turned off. The trace p was recorded in the pixel at the anomalous pacemaker (open circle indicated by "P" in the map and the sketch), where the excitatory waves first appeared. The arrowheads on trace p indicate the "pacemaker potential"-like diastolic depolarization prior to the action potential. This "pacemaker potential"-like diastolic depolarization was observed only around the anomalous pacemaker. As shown in the map (Fig. 6b), the excitatory waves spread toward the surrounding area from the anomalous pacemaker during the events of BIS.

The site of the anomalous pacemaker was not stable during the events of BIS. Figure 7 shows two maps of excitation spread obtained in the same event of BIS with an interval of about 2 min. These maps were obtained 1.5 h after the application of Bleb (50 μM). The sites of the anomalous pacemakers are different in these two maps, indicating that the site of the anomalous pacemaker can spatially shift during an event of BIS. Such "pacemaker shifts" were observed in two of the six preparations of which the excitation spread patterns of BIS were mapped.

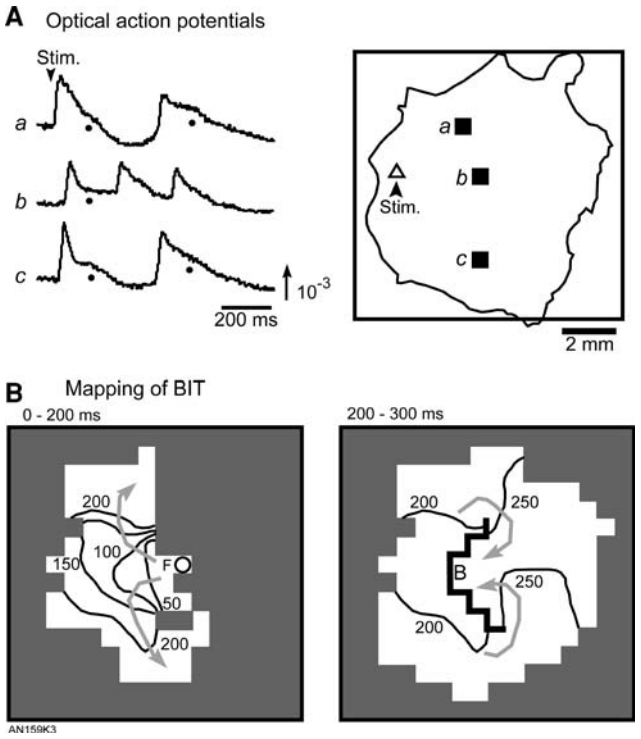

Fig. 5 Optical recording of BIT with a complex excitation spread pattern. **a** Optical signals of an event of BIT recorded at 2 h after the application of Bleb (10 μM). Optical action potentials simultaneously detected by three elements (*a*, *b*, and *c*) of the photodiode array are shown. *Right* Sketch of the preparation illustrating the location of the photodiode array on the preparation. *Open triangle with Stim* Position of the stimulation. **b** Maps of the excitation spread for the second optical action potentials. The timing of the appearance of the second action potential at the *anomalous* focus is regarded as time 0. Sequential maps during the BIT shown in **a** are represented. The positions of the wavefronts are displayed as *isochrone curves* at 50-ms intervals. *Gray arrow lines* Pathways of the excitation spread, *bold line indicated by B in the right map* functional blocked area over which the excitation cannot conduct. Other symbols are as defined in Figs. 1, 3, and 4

Combination pattern of BIS and BIT

As a rare case, BIT occurred in combination with BIS. This phenomenon was observed in the same preparation as that shown in Figs. 3 and 4. After the series of experiments on BIT shown in these figures, we switched off the pacing stimulation (about 2.2 h after the application of Bleb). Spontaneous optical action potentials then appeared with a very slow rhythm. Because the spontaneous action potentials appeared continuously, we considered that these electrical activities belonged to the category of BIS, although the rhythm was very slow. Figure 8 shows an example of this complex pattern.

Figure 8a shows simultaneously recorded action potential-related optical signals obtained from three pixels (a–c). A sketch of the preparation imaged on the photodiode array and the positions of the individual elements in the matrix

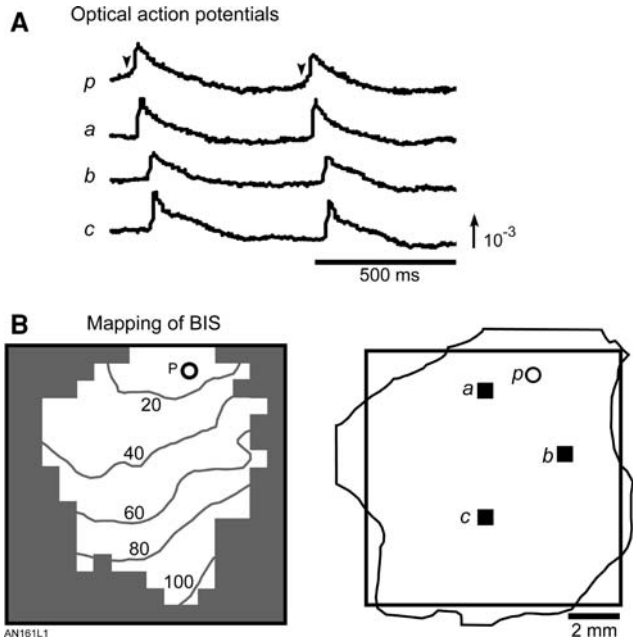

Fig. 6 Bleb-induced spontaneous electrical activity (*BIS*). **a** Optical action potentials detected simultaneously from four different positions (*p*, and *a–c*). The optical action potential first appeared in trace *p*, indicating the site of the anomalous pacemaker. The *arrowheads* in trace *p* indicate the "pacemaker potential"-like diastolic depolarization. This event of BIS was observed 2 h after the application of Bleb (50 μM). **b** Optical mapping of the excitation spread. The excitatory wave spread toward the surrounding area from the anomalous pacemaker (*open circle indicated by P*). Other symbols are as defined in Figs. 1, 3, and 4

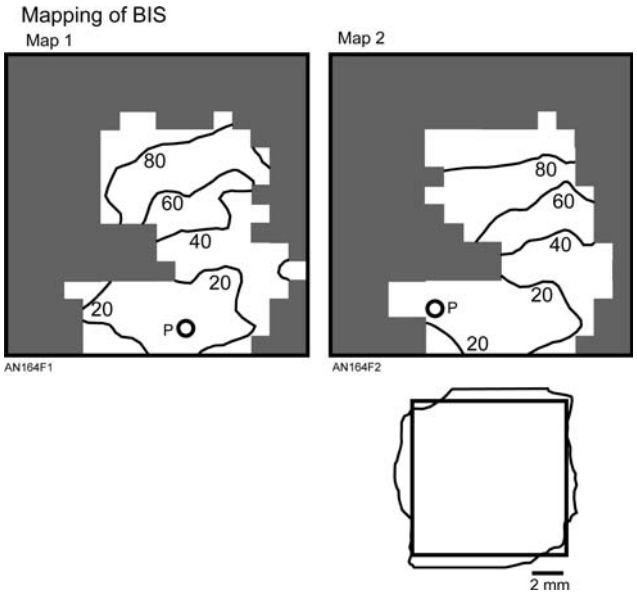

Fig. 7 Pacemaker shift during an event of BIS. Two maps of excitation spread obtained in the same event of BIS with an interval of about 2 min are shown. The data were obtained 1.5 h after the application of Bleb (50 μM). Between the two maps, the sites of the anomalous pacemakers are different. Other symbols are as defined in Figs. 1, 3, 4 and 6

array are shown on the right. Two action potentials were recorded during the event in traces a and b, whereas only one action potential was recorded in trace c.

The maps of excitation spread during the event are represented in Fig. 8b. The left map shows the excitation spread of the first action potentials. The excitation first appeared at an anomalous pacemaker positioned near the lower edge of the recording area, subsequently propagating upward. The excitation spread of the second action potential is shown in the right map. In this map, the spatial pattern of excitation spread was somewhat complex. About 145 ms after the first action potentials, the excitatory wave appeared from an anomalous focus on the right (open circle indicated by "F1"), subsequently spreading leftward. Shortly (about 20 ms) after the appearance of this focus, the second anomalous focus appeared on the left ("F2"), and the excitation spread rightward. The two excitatory waves collided in the upper part of the recording area. Note that the second action potentials spread only in a restricted region in the upper part of the recording area and that this pattern resulted in the difference in the number of action potentials between traces a and b and trace c. Because it is

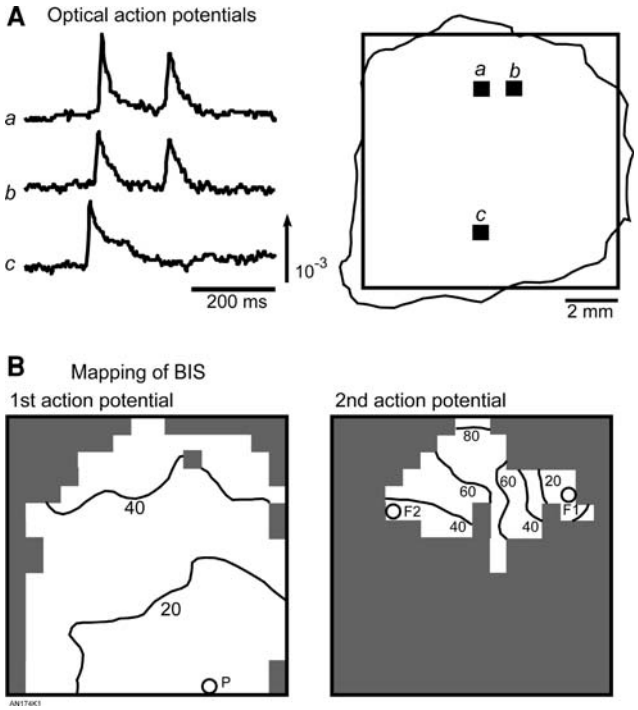

Fig. 8 Combination pattern of BIS and BIT. **a** Optical action potentials detected simultaneously from three different positions (*a–c*). This event of BIS was observed 2 h after the application of Bleb (100 μM). **b** Optical mapping of the excitation spread. The first action potential spread toward the surrounding area from the anomalous pacemaker near the lower edge (*left map*). The second action potential appeared from the two anomalous foci indicated by *F1* and *F2* and spread in the limited area of the preparation (*right map*). Other symbols are as defined in Figs. 1, 3, 4, and 6. See text for details

plausible that the second action potentials were triggered by the spontaneous first action potentials, we consider this event to be a combination pattern of BIS and BIT. This combined pattern was observed only in this preparation.

Discussion

In the study reported here, we have demonstrated, for the first time, the anomalous electrical activities induced by Bleb and analyzed their spatiotemporal pattern of excitation spread. Bleb is a recently identified selective and specific small molecule inhibitor of the ATPases associated with class II myosin isoforms in an actin-detached state [8, 11, 12]. The suppression of muscle contraction by Bleb application seems to be due to the inhibition of the ATPase activities of cardiac myosin, which results in the suppression of sliding of the myosin and actin chains [13, 14]. Efimov and his colleagues were the first to introduce Bleb as an excitation–contraction uncoupler for use in the optical monitoring of action potentials [8, 9]. Using Bleb, they successfully mapped the excitation spread in Langendorff-perfused or isolated rabbit hearts and isolated human A-V nodal preparations. Here, we also prove the effectiveness of this chemical in isolated rat atrium preparations. As shown in Figs. 1 and 2a, the suppression of the muscle contraction was so strong that the contraction-related optical signals were often eliminated almost completely. It should be noted that Efimov's group recorded optical action potentials using a fluorescence voltage-sensitive dye (di-4-ANEPPS), whereas we used an absorption dye (NK2761). Because the optical signals are much more sensitive to the muscle contraction in the absorption recording than in the fluorescence recording, the reduction of the contraction-related optical signals (artifacts) is a more serious problem. On the other hand, this chemical did not affect the optical action potentials, as shown in Fig. 2b. Bleb would therefore seem to be an ideal chemical for the suppression of contraction-related optical signals.

However, we unexpectedly encountered the anomalous electrical activities induced by the application of Bleb. No such anomalous electrical activities were observed when we used BDM or Cyto D for the suppression of contraction-related optical signals in isolated rat atrial preparations (data not shown). Although Efimov's group used Bleb at concentrations of 0.1–10 μM, we carried out experiments at higher concentrations (10–100 μM) because we wanted to obtain a stronger suppression of contraction due to the high sensitivity of the optical signals to the contraction artifacts, as stated above. As can be seen in Fig. 2a, the time courses of the suppression of contraction-related optical signals were almost superimposable between those of 10 and 100 μM. In addition, using mouse papillary

Table 1 Incidence of blebbistatin-induced anomalous electrical activities

Concentration of blebbistatin (μM)	Type of anomalous electrical activities				Total number of preparations tested
	Only BIT	Only BIS	Both	None	
10	0	0	1	1	2
20	0	0	0	3	3
50	0	3	1	2	6
100	2	1	1[a]	0	4

BIT, Blebbistatin (Bleb) -induced triggered activity; BIS, Bleb-induced spontaneous electrical activity

[a] Spontaneous activity with long intervals

muscle preparations, Dou et al. demonstrated that the contraction was almost completely suppressed by the application of 10 μM Bleb [13]. The concentration of 10 μM may be the saturation dose for the suppression of contraction. On the other hand, as shown in Table 1, the incidence of anomalous electrical activities seems to be higher when the concentration of Bleb is high. The difference in the concentration of Bleb used by Efimov's group and in our study seems to be one of the reasons for our finding of anomalous electrical activities that they did not observe. The difference in animal species (rabbit and rat) may also contribute to this difference. Nevertheless, the event of BIT shown in Fig. 5 was observed in the presence of 10 μM Bleb, indicating that this concentration is not low enough to avoid the generation of anomalous electrical activities in the isolated rat atrium preparation.

It has been suggested that anomalous intracellular calcium dynamics cause anomalous electrical activities, such as triggered activities and anomalous excitation [15–18]. It is plausible that the BIT and BIS reported in this study are the result of disturbed intracellular calcium dynamics induced by Bleb via unknown mechanism(s) as it is known that Bleb inhibits muscle contraction by disturbing the normal interaction of the myosin and actin chains. The fact that BIT and BIS appeared 1.5–2 h after the application of Bleb, when Bleb strongly suppressed the muscle contraction, supports this hypothesis.

However, Efimov's group excluded this possibility of disturbed intracellular calcium dynamics in an experiment using a calcium indicator (fluo-5F) and isolated rat ventricular cells [8]. They showed that, although the shape of intracellular calcium transient was not affected by the application of Bleb, the resting fluorescence increased in a dose-dependent manner (see Figs. 1A and C in Ref. [8]). These authors suggested that this increase resulted from the photosensitivity of Bleb in the UV range. Using a two-photon confocal analysis of indo-1 fluorescence, Farman et al. showed that a low dose of Bleb (0.5 μM) did not increase the resting intracellular calcium concentration in isolated rat cardiac myocytes [14]. Given this result and those of our study in which higher concentrations of Bleb were used, we suggest that an increase in resting calcium concentration and/or disturbance of intracellular calcium dynamics cannot be ruled out. This possibility is supported by our observation of abnormal electrical activities in which a disturbance of intracellular calcium dynamics may play a part (see also Refs [3–6]).

In other words, our relatively high concentration of Bleb disturbed the normal intracellular calcium dynamics and/or increased the resting level of intracellular calcium ions by some unknown mechanism(s), resulting in the generation of anomalous electrical activities, such as BIT and BIS. We optically recorded the anomalous electrical activities (triggered activities and spontaneous excitation with an ectopic pacemaker) evoked by the increase of intracellular calcium ion using the calcium ionophore (A23187) in isolated rat atrial preparations (N.K. and T.S., unpublished results).

In this study, we were able to demonstrate the spatiotemporal dynamics of triggered activities using optical techniques. As shown in Fig. 4b–d, when triggered activity occurred, the second optical action potential first appeared at the anomalous focus and then spread to the limited area of the preparation. In addition, the position of the focus and the area of excitation spread varied among the events of the triggered activities. The meandering of the focus seems to be the result of spatiotemporal inhomogeneity of the intracellular calcium dynamics which evoke triggered activities. Instability of the intracellular calcium dynamics would give rise to multiple foci in the triggered activity, as shown in Fig. 8b. The variation in the area of excitation spread may be due to the spatiotemporal fluctuation of the refractoriness.

Optical methods also revealed that, in the case of spontaneous excitation (BIS), the excitatory waves were generated at anomalous pacemaker sites. At the site of the pacemaker, the "pacemaker potential"-like diastolic depolarization was observed. The position of the pacemaker was often stable, but it sometimes shifted. This "pacemaker shift" also seems to be the result of fluctuation of the intracellular calcium dynamics, which influences the slope of "pacemaker potential"-like depolarization. The combination pattern of BIS and BIT

shown in Fig. 8 suggests that BIS and BIT are generated by somewhat different mechanisms because the anomalous pacemaker of BIS and anomalous foci of BIT are different. Consequently, BIS is not a simple repetition of BIT.

In earlier studies in which optical methods were used, we observed the generation of the circus movement of the excitatory wave and the appearance of ectopic pacemaker(s) in isolated atrial preparations, which were evoked by repetitive stimulation [3, 4, 6]. It has been suggested that elevation of the intracellular concentration of calcium ion due to repetitive stimulation contributes to the generation of these phenomena. In our study, disturbances of the intracellular calcium dynamics did appear to contribute to the generation of BIT and BIS. In all our our experiments, spatiotemporal inhomogeneity of the intracellular calcium dynamics seems to have played an important role in the generation of arrhythmia and anomalous electrical activities. An intracellular Ca^{2+}-imaging study aimed at analyzing these phenomena and elucidating the mechanism(s) of the disturbance of the intracellular calcium dynamics would be helpful. To this end, we are now preparing to set up a combined optical recording system for voltage and Ca^{2+} imaging.

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