Esmolol is antiarrhythmic in doxorubicin-induced arrhythmia in cultured cardiomyocytes – determination by novel rapid cardiomyocyte assay

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Abstract Cardiac toxicity is an uncommon but potentially serious complication of cancer therapy, especially with anthracyclines. One of the most effective anticancer drugs is doxorubicin, but its value is limited by the risk of developing cardiomyopathy and ventricular arrhythmia. When applied to a network of periodically contracting cardiomyocytes in culture, doxorubicin induces rhythm disturbances. Using a novel rapid assay based on non-invasive ion-conductance microscopy we show that the β-antagonist esmolol can restore rhythm in doxorubicin-treated cultures of cardiomyocytes. Moreover, esmolol pre-treatment can protect the culture from doxorubicin-induced arrhythmia.

Key words: Arrhythmia; Doxorubicin; Esmolol; Cardiomyocyte; Rapid assay

1. Introduction

Despite its well-documented cardiotoxic effects, the anthracycline antibiotic doxorubicin (adriamycin) remains a major and most effective anticancer agent against human malignancies such as leukaemia, lymphomas and many solid tumours [1]. Doxorubicin is also used in combination with other chemotherapeutic agents (platinum and paclitaxel) for systemic treatment of patients with endometrial cancer [2]. In prostate cancer anthracyclines are often applied as a monotherapy or in combination with other drugs [3]. However, chronic administration of doxorubicin produces a cumulative dose-dependent and irreversible cardiac toxicity and arrhythmia [4] that limits its optimum use. Wang showed electrocardiogram analysis of doxorubicin cardiac toxicity in 160 cancer cases. Many patients displayed abnormal electrocardiograms [5]. Raabe and Storstein reported that the number of ventricular extrasystoles increased after doxorubicin infusion [6].

Cellular and molecular mechanisms of doxorubicin toxicity are not fully understood. Available laboratory evidence suggests that an increase in oxidative stress, brought about by increased free radical production and decreased myocardial endogenous antioxidants, plays an important role in the pathogenesis of heart failure. Adriamycin-induced apoptosis and hyperlipidaemia may also be involved in the process [7].

Esmolol is an ultra short-acting intravenous cardioselective β-antagonist and it has been shown to produce a significant dose-dependent decrease in ventricular rate [8]. Esmolol is indicated in short-term treatment of hypertension and tachycardia during the perioperative period and in clinical situations that require rapid β-receptor blockade [9]. The efficacy and safety of esmolol has been proven in specific clinical settings, i.e. in patients with hypertension, unstable angina, myocardial infarction, and supraventricular tachyarrhythmias. As esmolol's antiarrhythmic action is at the level of the β-adrenergic receptor, we hypothesised that it could be effective in correction of doxorubicin-induced arrhythmia.

To study this possibility we chose a neonatal rat cardiomyocyte culture. This culture provides a unique in vitro model for studying drug effects on the rhythm of cardiomyocyte contraction. When cultured as a network, the early culture consists of single myocytes, which exhibit unsynchronised contraction. When any two spontaneously contracting neonatal myocytes establish contact, they synchronise. Subsequently the cells proliferate, migrate and assemble into a monolayer network that beats synchronously. Previously, Lampidis and co-workers showed that doxorubicin produced arrhythmias in cultures of neonatal rat cardiomyocytes [10]. Our doxorubicin-related data discussed in the present paper and obtained using a novel and rapid assay with a scanning ion conductance microscope [11] are in good agreement with this report.

2. Materials and methods

2.1. Preparation of a primary culture of neonatal rat cardiomyocytes

Ventricular myocytes were isolated from the hearts of 1–2 day old rats [12]. Cells were kept in Dulbecco's modified Eagle's minimum essential medium with 5% foetal calf serum (v/v), 200 μg/ml streptomycin, 200 U/ml penicillin, 5% (v/v) non-essential amino acids. Geneticin (G418) at 50 μg/ml was added to inhibit fibroblast growth. (All reagents are from Gibco, Paisley, UK.) Cells were maintained at 37°C in an atmosphere of humidified air plus 5% CO_2. The density of plating was 500 000 cells/ml. Cells were used following 3–4 days culture on glass coverslips (small network of cardiomyocytes).

2.2. Immunocytochemical detection of α-actinin

Cells were fixed with 4% formalin solution in phosphate-buffered saline (PBS), permeabilised with 0.1% Triton X-100 in PBS for 20 min, blocking solution of 10% normal goat serum was applied, followed by monoclonal antibodies to sarcomeric α-actinin, clone EA-53 (Sigma-Aldrich, Poole, UK, A7811) in 1:100 dilution. The antigen was de-

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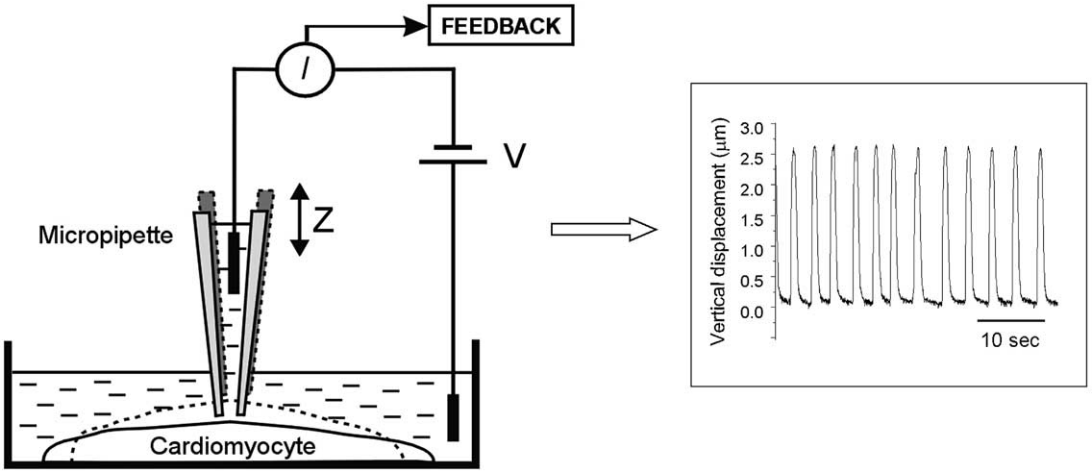

Fig. 1. A diagram of the experimental apparatus for recording of vertical displacement of the cell surface of a cardiomyocyte during contraction.

tected using fluorescein-conjugated secondary antibody. Slides were observed under a Nikon-Eclipse TE300 microscope.

2.3. Drugs

A 100 μg/ml stock solution of esmolol hydrochloride (Brevibloc, Gensia Europe Ltd, Bracknell, UK) in Hanks' balanced salt solution buffer (Gibco) was prepared on the day of the experiment. Doxorubicin (Sigma-Aldrich) was stored at −20°C as a 250 μg/ml stock solution in Hanks' balanced salt solution buffer. All drugs were diluted to the desired concentrations in Hanks' balanced salt solution buffer on the day of the experiment.

2.4. Effect of drugs on amplitude and rhythm of contraction of cardiomyocytes

To investigate the changes of the rhythm of cardiomyocyte contraction caused by the addition of drugs, we recorded the vertical cell displacement of individual cells using a scanning ion conductance microscope as described previously [11]. Fig. 1 shows our experimental setup. Briefly, the sensitive probe of the scanning ion conductance microscope is a glass micropipette, filled with PBS solution. The Ag/Cl electrode is plugged in to the pipette and connected to a current amplifier to measure the ion current that flows through the tip of the micropipette. The current provides a feedback signal to move the micropipette up and down, in order to keep the pipette–cell membrane separation constant during contraction. Therefore, as the cell surface rises during contraction, the micropipette is displaced vertically (Z position in Fig. 1). This measurement allows alterations in contraction to be recorded (inset).

3. Results

To establish the proportion of cardiomyocytes in the geneticin-treated cultures, cells were stained with antibodies to detect sarcomeric α-actinin. Immunodetection showed that almost 80% of the cells possessed the myocyte marker, α-actinin (Fig. 2).

Under control conditions, the cells beat regularly, with little variation in rate as measured by recording the vertical cell surface displacement of individual cells. We expressed the rate as beat to beat interval to facilitate arrhythmia analysis. This, clearly, also varied little and in each study beat to beat intervals were consistent in each preparation – each roughly near 3 s beat to beat interval. In this setting we applied doxorubicin, alone or together with esmolol. Control 5 day old cultures displayed regular contractions (Fig. 1, inset). In the first set of experiments ($n = 5$) chronic addition of doxorubicin in low doses (0.1 μg/ml, every 48 h for 5 days) induced arrhythmic activity. These rhythm irregularities included premature beats with compensatory pauses, sometimes with a bigeminal pattern (Fig. 3A). Fig. 3B shows the frequency histogram of the inter-beat intervals. The S.D. is wide, reflecting the wide variation in inter-beat interval (arrhythmia). Doxorubicin-treated cultures have beat intervals from 3 to 7 s and their S.D.s range from 1.4 to 3.9. The peak is diffuse because the premature beats have a short interval, and they are also associated with a long post-beat compensatory pause, i.e. long interval. Having induced arrhythmia, we applied 1 μg/ml esmolol acutely to these cultures. Esmolol restored rhythmic contraction as shown in Fig. 3C. The histogram in Fig. 3D has only one narrow peak – no bigemini and few, if any, premature beats. The pattern in this representative figure was consistent, and was demonstrated in every experiment. Esmolol-treated cultures have beat intervals from 1.4 to 2.2 s and their S.D.s range from 0.3 to 0.7.

In the second set of experiments ($n = 6$) a high dose of doxorubicin (100 μg/ml) given acutely caused arrhythmia in 5 day old untreated cardiomyocytes with an occasional bigeminal

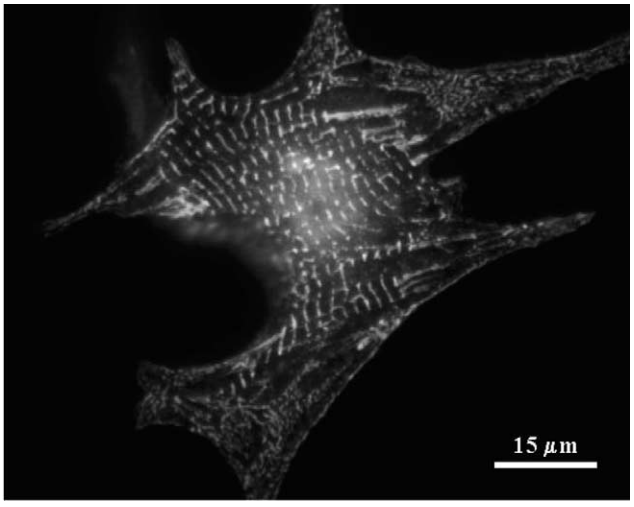

Fig. 2. Immunostaining of a cardiomyocyte cluster for cytoskeletal proteins that are specific to myocardium (α-actinin).

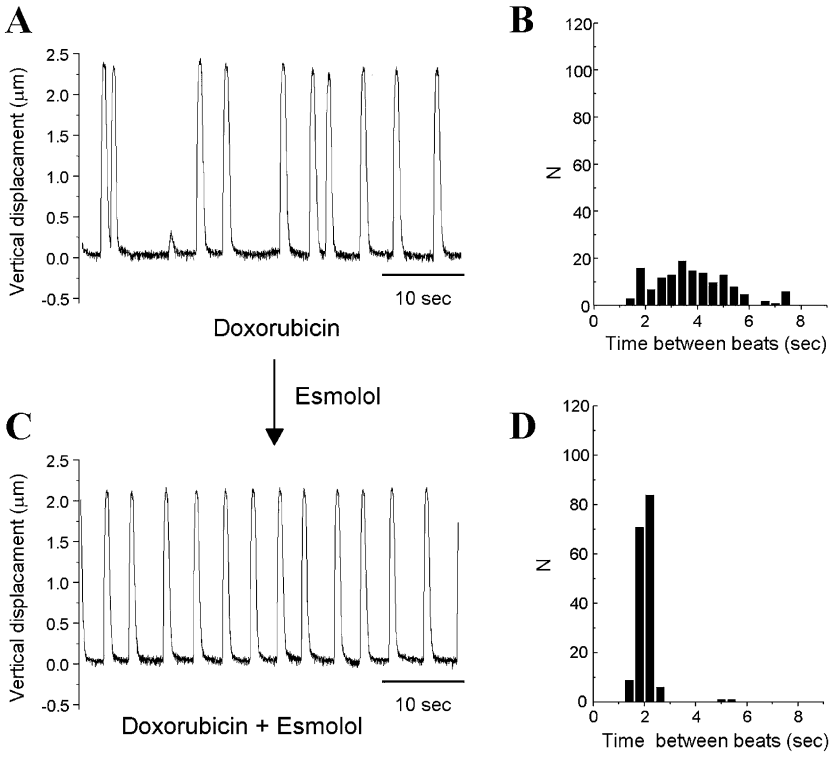

Fig. 3. Typical example of the effect of esmolol on arrhythmia produced by chronic administration of low dose doxorubicin. A: Doxorubicin induced rhythm disturbances, when added in low concentration (0.1 μg/ml) over 5 days. B: Histogram of intervals of time between beats of cardiac cell culture treated with doxorubicin at 0.1 μg/ml over 5 days. Time of recording = 10 min. Time between all beats = 3.9 s, ±1.6 (mean ± S.D.). *N*, number of intervals. C: Esmolol (1 μg/ml) restored rhythm in doxorubicin-treated cultures. Measurement was taken 10 min after addition of esmolol. D: A histogram of intervals of time between beats of cardiac cell culture treated with doxorubicin at 0.1 μg/ml over 5 days after addition of esmolol (1 μg/ml). Time of recording = 10 min. Time between beats = 2.0 s, ±0.4 (mean ± S.D.). Note large S.D. in B compared with D.

pattern. The arrhythmia appeared as soon as 3–5 min after doxorubicin addition (data not shown, but similar to data shown in Fig. 3A). One hour later a severe arrhythmia was evident in these cultures: the cells had prominent bigemini (Fig. 4A). As in the previous set of experiments, the S.D. was broad and there were two peaks reflecting variable arrhythmic inter-beat intervals, with an early peak for bigemini (Fig. 4B). Doxorubicin-treated cultures have beat intervals from 3 to 5 s and their S.D.s range from 1.8 to 3.0. Pre-treatment with esmolol (1 μg/ml) added 30 min before doxorubicin addition prevented the arrhythmic effect of the drug. Even after 60 min of exposure to doxorubicin there were still no disturbances of the rhythm of cardiomyocyte contraction (Fig. 4C). The histogram now shows one peak (Fig. 4D). Esmolol-pre-treated cultures have beat intervals from 2.0 to 2.8 s and their S.D.s range from 0.6 to 0.9.

4. Discussion

Neonatal rat cardiomyocyte cell culture has been developed to address various aspects of myocardial physiology [10,13,14]. Our assay, using this type of model, allowed us to rapidly assess the action of drugs applied to a culture of myocytes. Recordings of single living cell contractions in normal physiological solutions can be obtained in real time and sufficient data for statistics can be quickly collected from the same culture. Our assay has been used previously to show that the time course of a cardiomyocyte contraction is commensurate with its Ca^{2+} transient [11].

The cardiomyocyte culture model has some limitations since it is based on a layer of not fully differentiated cells. Electrophysiologically they have no specialised pacemaker or conducting cells as found in whole heart. Mechanically contraction is constrained due to attachment of the cells to the surface on which they are cultured, and not constrained by pressure or volume loads. However, it provides the advantages of single cell study with the simultaneous convenience of the manipulation and study of a two-dimensional network of neighbouring cells synchronously contracting. In particular its two-dimensional nature allows the application of different microscopy techniques. Moreover, despite remarkable differences to whole heart, it can show arrhythmic behaviour, commensurate in some respects with that in whole heart, in response to arrhythmic and antiarrhythmic agents. A variety of studies have therefore been performed on this model. For instance, it has been possible for the first time to look at the influence of the bile acid taurocholate on the function of cardiomyocytes [15,16].

Previously the mechanism of doxorubicin cardiac toxicity was shown to implicate Ca^{2+} homeostasis as a primary triggering mechanism that leads to cardiomyopathy [17]. Maeda et al. showed that ventricular rat cardiac myocytes treated with doxorubicin exhibit significant changes in Ca^{2+} transients. The parameters of these Ca^{2+} transients showed strong

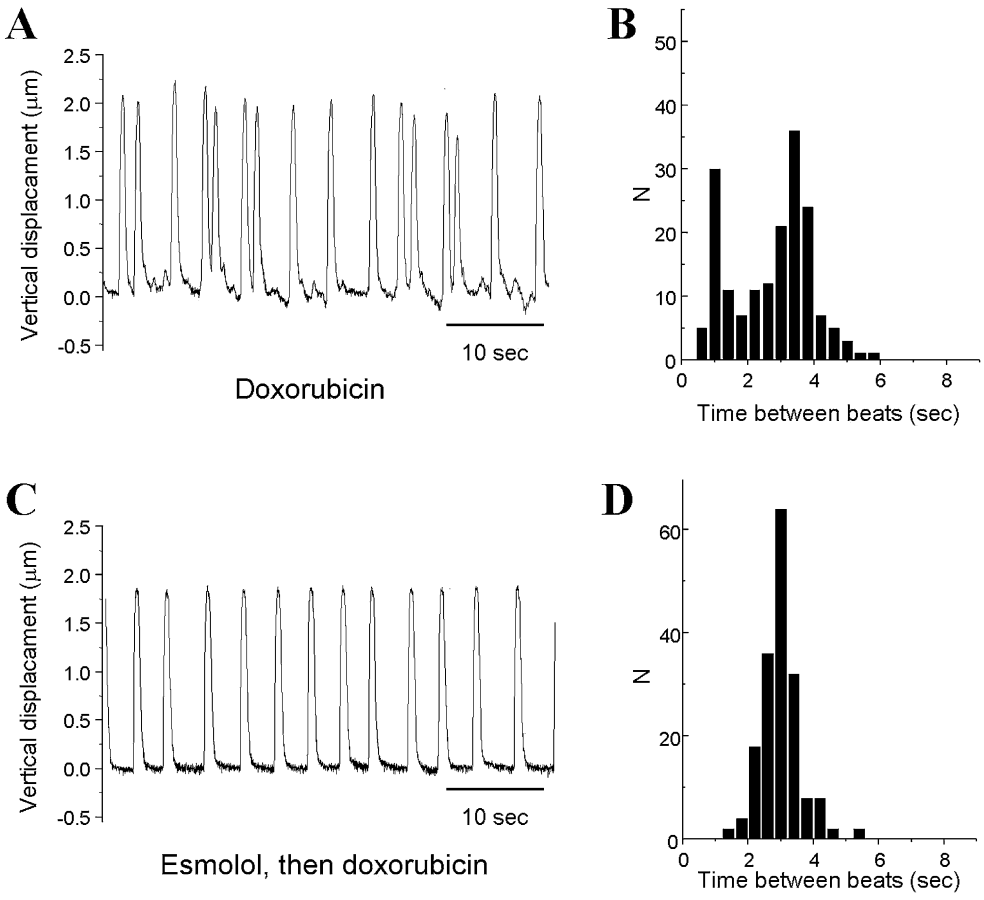

Fig. 4. Typical example of how esmolol prevents arrhythmia induced by a high dose of doxorubicin. A: Doxorubicin induced significant rhythm disturbances, when added in high concentration (100 μg/ml). Measurement was taken 60 min after doxorubicin addition. B: Histogram of intervals of time between beats of cardiac cell culture treated with doxorubicin at 100 μg/ml. Time of recording = 10 min. Time between beats = 2.7 s ± 1.2 (mean ± S.D.). C: Pre-treatment with 1 μg/ml esmolol added 30 min before doxorubicin preserved the initial regular rhythm of cardiomyocytes. D: Histogram of intervals of time between beats of cardiac cell culture pre-treated with 1 μg/ml esmolol and after treatment with 100 μg/ml of doxorubicin. Time of recording = 10 min. Time between beats = 2.5 s ± 0.6 (mean ± S.D.). Note large S.D. in B compared with D.

β-adrenergic responsiveness [18]. Although the precise mechanisms of action and interactions of esmolol and doxorubicin are largely unknown, we can speculatively implicate a β-adrenoceptor responsiveness of the cardiomyocyte's calcium transient parameters. There is a likely interaction between doxorubicin toxicity and esmolol's β-adrenergic blocking action.

Interestingly, esmolol has the effect on a cultured myocyte system where there are no catecholamines to block. A similar non-catecholamine-blocking effect was found for another β-blocker, the $\beta_2$adrenoceptor blocker ICI 118,551 in cardiomyocytes. A G_i-dependent pathway was proposed for the negative inotropic action of the β = blocker [19].

We conclude that in this model of cultured cardiomyocytes esmolol pre-treatment can prevent doxorubicin arrhythmic action and esmolol can also reverse doxorubicin-induced arrhythmia. Moreover, although we did not study the molecular mechanisms of the compounds under investigation, our studies strongly suggest that our simple non-animal model can be used for investigation of individual arrhythmogenic drugs and their interactions.

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