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Cardiotoxic Effects of Adrenochrome and Epinephrine on the Mouse Cultured Myocardium

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ABSTRACT—Effects were studied on cultured mouse ventricular myocardial cells of 10^{-6}–10^{-3} M epinephrine and adrenochrome, a metabolite of epinephrine. Single ventricular myocardial cells in culture had negative chronotropic responses to adrenochrome (10^{-4} M) and positive responses to epinephrine (10^{-6} M). However, cultured ventricular myocardial cells in confluent sheets had positive chronotropic responses to adrenochrome (2×10^{-4} M) and epinephrine (10^{-6} M). Cumulative LDH leakage from ventricular myocardial cells in confluent sheets increased remarkably after application of 10^{-3} M adrenochrome but only somewhat after application of 10^{-3} M epinephrine. Both adrenochrome and epinephrine (10^{-6} M) induced mitochondrial fusion and an increase of atrial specific granules, in ventricular myocardial cells in confluent sheets. Epinephrine induced an increase in size of myofibrils. In cultured ventricular myocardial cells, adrenochrome at 2×10^{-4} M induced mitochondrial damages, as shown by a swollen appearance, but epinephrine never induced such necrotic changes even at concentrations up to 10^{-3} M. The role of adrenochrome and epinephrine in catecholamine cardiotoxicity is discussed.

INTRODUCTION

Myocardial necrosis which occurs during reperfusion following myocardial ischemia might be, at least in part, due to excessive release of catecholamines in the heart [17, 21, 26]. Administration of excessive concentrations of epinephrine, norepinephrine or isoproterenol has been shown to induce myocardial necrosis in experimental animals [4, 5, 20, 22]. Furthermore, adrenochrome is known as one of the non-physiological products of epinephrine [8, 19], but the *in vivo* formation of adrenochrome has been demonstrated in pathological conditions [1, 14, 15]. Yates and his coworkers [29] assumed that in isolated perfused rat hearts the drug produced cell membrane injury and contractile impairment and might be partly responsible for necrogenesis in catecholamine-induced cardiomyopathy. On the contrary, Wheatley and his colleagues [28] insisted that the catecholamine-induced myocardial cell damage in isolated rat hearts and papillary muscles was the result of epinephrine and norepinephrine stimulation, but not due to adrenochrome, since 10^{-4} M of adrenochrome was required in order to produce a cardiotoxic effect, while the physiological peak concentration of epinephrine was 10^{-8} M.

The cultured myocardium has not been employed to evaluate the effects of adrenochrome and epinephrine on myocardial cells in catecholamine-induced cardiotoxicity. In the present study, we used mouse cultured myocardial single cells and confluent sheets to examine the effects of the drugs on beat rates and ultrastructure in the preparations. We also examined the cumulative leakage of lactate dehydrogenase (LDH) from the cells in the confluent sheets to the culture medium, as an index of cellular damage [18].

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MATERIALS AND METHODS

Mouse ventricular myocardial cells were prepared as reported previously [16]. Single myocardial cells were prepared by seeding cells in plastic dishes (Falcon, ϕ35 mm) at concentrations of 2.5–5×10^4 cells/dish. Myocardial cells in a confluent monolayer were prepared by seeding cells in a 4 Well Multidish (Nunclon, Nunc) at concentrations of 1×10^5 cells/well or, for an ultrastructural study, in MicroWell Modules (Nunc) at concentrations of 1.6×10^4 cells/well.

Adrenochrome was synthesized at the University of Naples, as described by Sobotka and Austin [25]. Adrenochrome and epinephrine bitartrate (Sigma) were applied to the myocardial cells after cultivation for 5 days. The culture medium was replaced with Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) buffered with 10 or 20 mM BES (pH 7.3) 1–2 hr prior to the application of the drugs and the myocardial cells were then placed at 37°C in a CO_2-free environment. Adrenochrome (10^{-6}, 10^{-5}, 10^{-4}, 2×10^{-4} and 10^{-3} M) or epinephrine (10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M) dissolved freshly in the MEM was applied to the myocardial cells by replacing the normal MEM.

Beating rates of cultured single and confluent myocardial cells were obtained by counting beats in the screen image of individual myocardial cells recorded by a television microscope (a phase contrast inverted microscope: Nikon, TMD; a color camera: Hitachi, DK-7001N) coupled to a video system (Panasonic, AG-6720A). Statistical analyses were performed using the unpaired Student's *t*-test. Activity of LDH in the culture medium was measured spectrophotometrically according to Vassault [27]. An ultrastructural study was performed by a conventional electron microscopic method previously reported [16].

RESULTS

Chronotropic effects on single ventricular myocardial cells

Most of the single ventricular myocardial cells beat spontaneously at a variety of rates ranging from about 50 to 350 per min. Adrenochrome (10^{-6} and 10^{-5} M) did not induce chronotropic

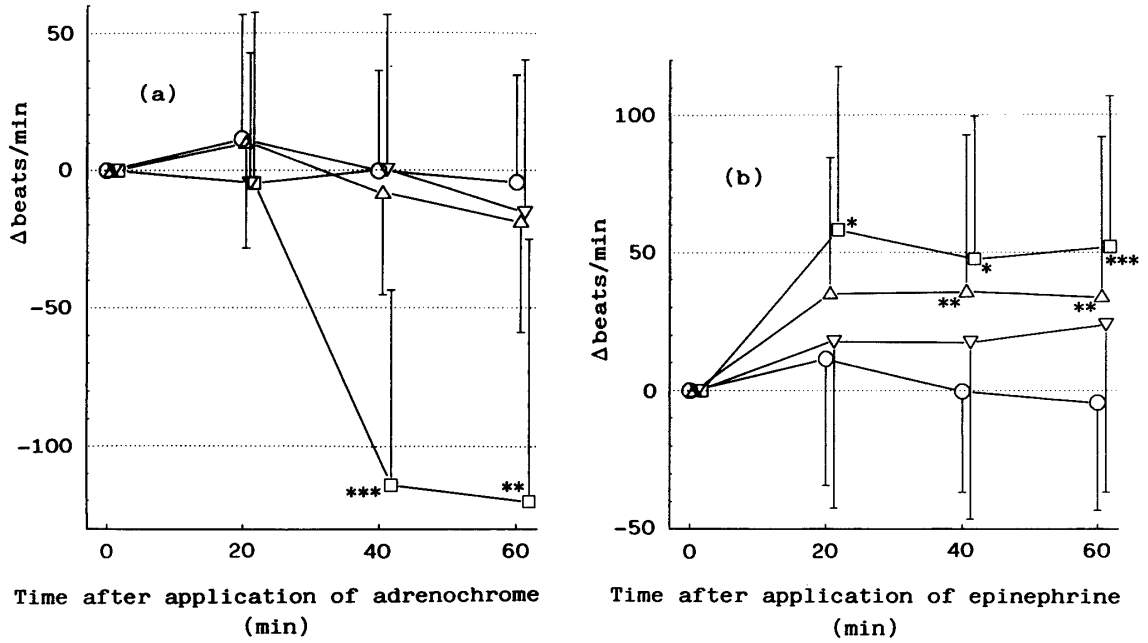


FIG. 1. Chronotropic effects of adrenochrome (a) and epinephrine (b) on single ventricular myocardial cells in culture. Graphs express changes in beat rates per min of the cells from the values before applying the drugs (means±S.D.). ○, control (a normal medium); △, 10^{-6} M; ▽, 10^{-5} M; □, 10^{-4} M. *, $P<0.01$; **$P<0.02$; ***$P<0.001$.

effects on single ventricular cells in a 60 min period after the onset of drug applications (Fig. 1a), though about half of the cells ceased beating in the normal medium after a 60 min exposure to a dosage of 10^{-5} M. A dosage of 10^{-4} M adrenochrome clearly exerted negative chronotropic effects at 40 min and 60 min after the onset of the application, but not at 20 min. At 40 min some cells ceased beating or died, and the beat rates of the beating cells decreased, statistically significant by Student's *t*-test (Fig. 1a, $P<0.001$ *vs.* control). At 60 min about half of the cells ceased beating or died, and the remaining cells beated weakly and slowly ($P<0.02$ *vs.* control). All of the cells died even when they were returned in the normal medium after a 60 min exposure to 10^{-4} M adrenochrome.

Epinephrine (10^{-6}, 10^{-5} and 10^{-4} M) induced positive chronotropic effects (Fig. 1b). There were statistical significances between the control value and the beat rates at 40 min ($P<0.02$) and 60 min ($P<0.02$) after the onset of application at a dose of 10^{-6} M, and at 20 min ($P<0.01$), 40 min ($P<0.01$) and 60 min ($P<0.001$) after the onset of application at a dose of 10^{-4} M. We never observed inhibitory effects of the dosages on the cells such as bradycardia or beat arrest.

Chronotropic effects on myocardial cells in confluent sheets

Ventricular cells in the confluent sheets appeared to beat synchronously and regularly. Adrenochrome (10^{-6} and 10^{-5} M) induced transient slightly positive chronotropic effects on the ventricular cells in confluent sheets at 1 hr after application (Fig. 2a). Adrenochrome at 2×10^{-4} M maintained the positive chronotropic effects over a period of 3 hr (Fig. 2a), the reverse of the case for single ventricular cells (Fig. 1a). The difference from the control value at 2 hr after application was statistically significant ($P<0.05$). At a higher dose (10^{-3} M) beats ceased immediately and completely.

Epinephrine (10^{-6}–10^{-3} M) effects on ventricular cells in confluent sheets had a tendency to shift the whole configuration of the graph toward the area of chronotropic positivity (Fig. 2b). Epinephrine induced no negative chronotropic responses up to 3 hr after the application.

LDH leakage

Cumulative LDH leakage from beating ven-

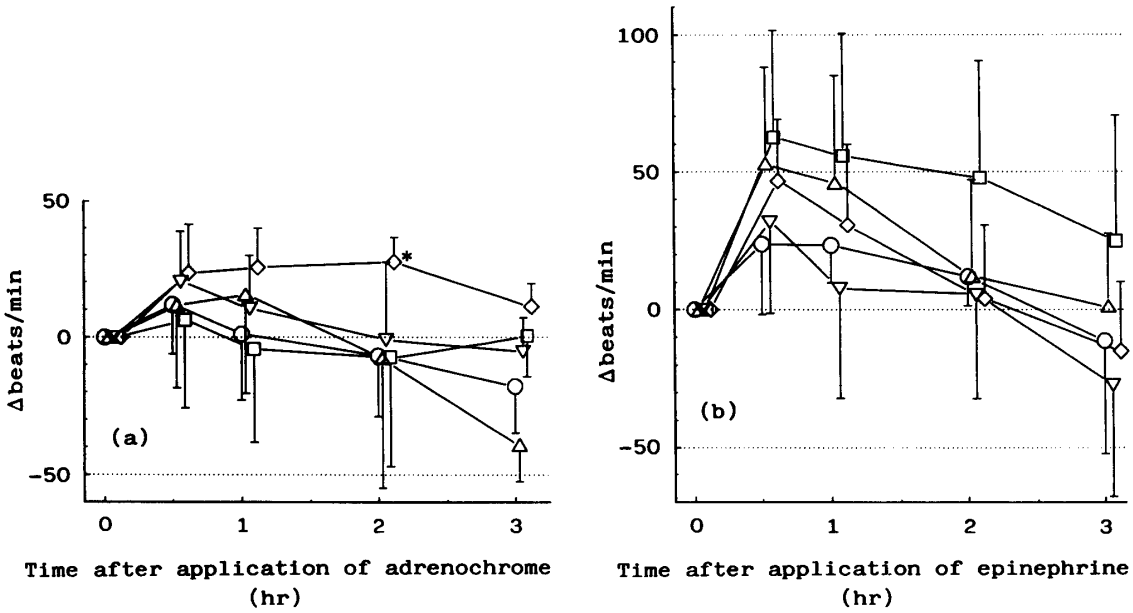


FIG. 2. Chronotropic effects of adrenochrome (a) and epinephrine (b) on cultured ventricular myocardial cells in confluent sheets. Graphs express changes in beat rates per min of the cells from the values before applying the drugs (means ± S.D.). ○, control; △, 10^{-6} M; ▽, 10^{-5} M; □, 10^{-4} M; ◇, 2×10^{-4} M (a) and 10^{-3} M (b). *: $P<0.05$.

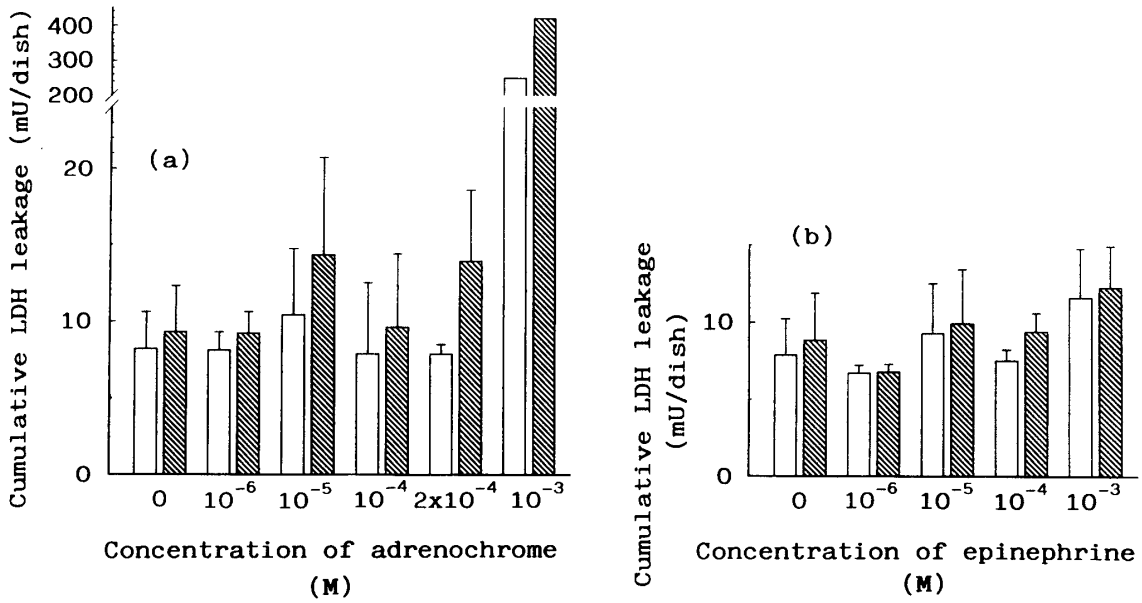


FIG. 3. Cumulative LDH leakage from cultured ventricular myocardial cells in confluent sheets after application of adrenochrome (a) and epinephrine (b) (means±S.D.). □ : 1 hr after application, ▨ : 2 hr after application.

tricular cells in confluent sheets was almost the same in a control experiment, 1 hr after application of 10^{-6}–2×10^{-4} M adrenochrome. However, LDH leakage was somewhat increased 2 hr after application of 10^{-5} and 2×10^{-4} M adrenochrome (Fig. 3a). Application of 10^{-3} M induced a significant increase of LDH leakage at 1 hr and 2 hr after the onset of the dosage (Fig. 3a), but not at 30 min (data not shown). These responses corresponded to the observation of the cell death accompanied with destruction of the cell membrane, as observed under a phase-contrast microscope.

Epinephrine (10^{-6}–10^{-3} M) induced no significant increase of LDH leakage from confluent ventricular cell sheets, though a dose of 10^{-3} M appeared to induce increase of the leakage somewhat (Fig. 3b). Indeed, we never observed cell death in epinephrine (10^{-6}–10^{-3} M) under a phase-contrast microscope.

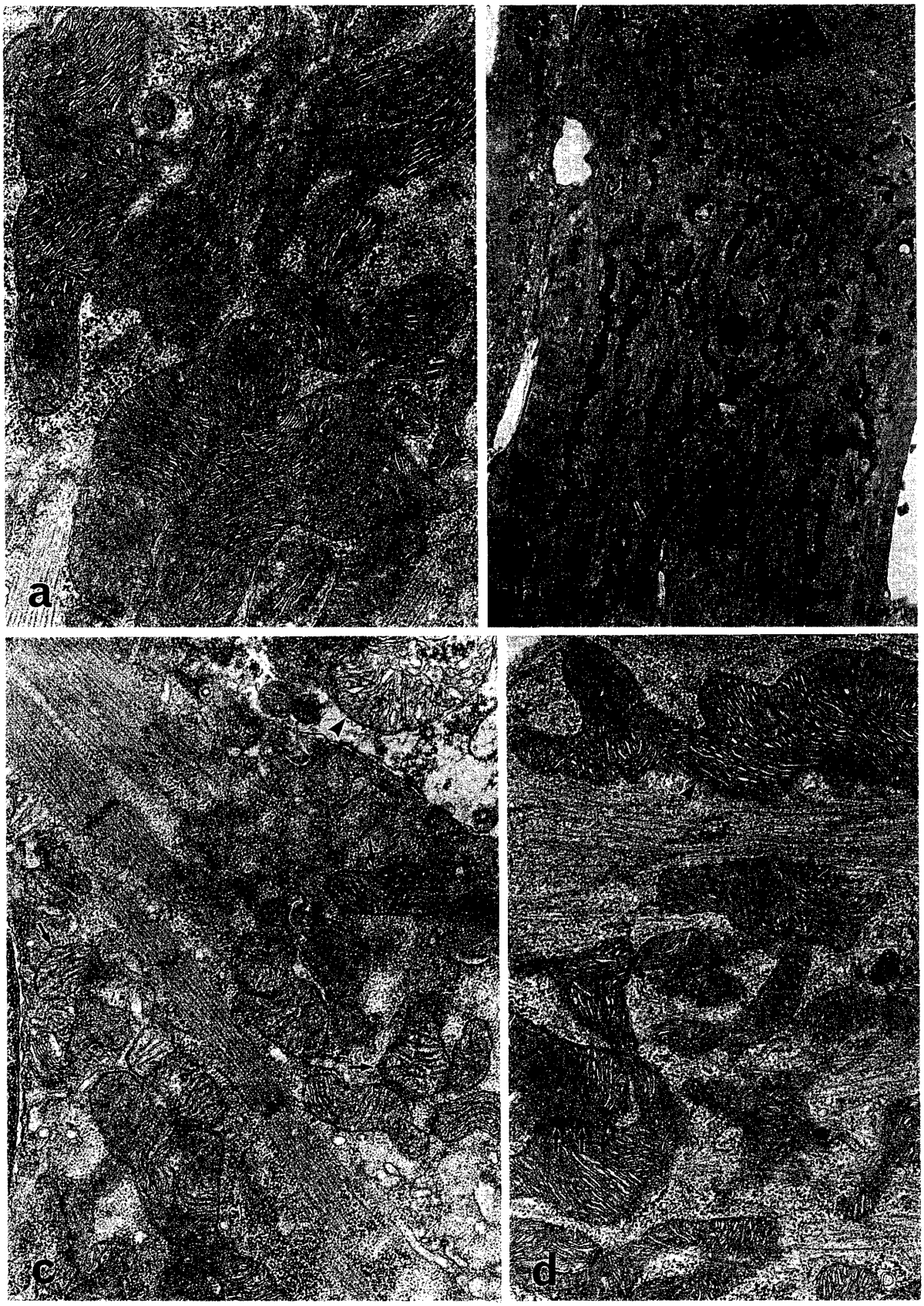
Ultrastructure

At 30 min after application of adrenochrome (10^{-6}–10^{-4} M) to ventricular cells in confluent sheets, mitochondria were already fusing (Fig. 4a). Atrial specific granules (atrial natriuretic peptide, [3, 12]) had increased, even among myofibrils, as well as in perinuclear regions (Fig. 4b). After a dosage of 2×10^{-4} M, mitochondria having more electron-lucent matrix and parallel cristae appeared abundantly (Fig. 4c; arrows). Furthermore, some mitochondria were markedly swollen and had electron-lucent matrix, vesicular cristae and amorphous deposits (Fig. 4c; arrow head). But, the specific granules were scarcely seen at this point (Fig. 4c). Glycogen granules and rough endoplasmic reticulum had increased in number around mitochondria (10^{-5}–2×10^{-4} M; Fig. 4a-c).

Application of epinephrine (10^{-6}–10^{-3} M) for 30 min induced mitochondrial fusion (Fig. 4d, arrows), giant mitochondria (Fig. 4d, arrow head), an increase in number of the atrial specific granules in perinuclear regions and among myofibrils (Fig. 4e, f, arrows) and an increase in size of myofibrils (Fig. 4e) in a dose-dependent manner, but no mitochondrial changes such as seen after the application of 2×10^{-4} M adrenochrome (Fig. 4c).

DISCUSSION

The response of single ventricular myocardial cells to adrenochrome was different from that of the ventricular cells in confluent sheets; first, the



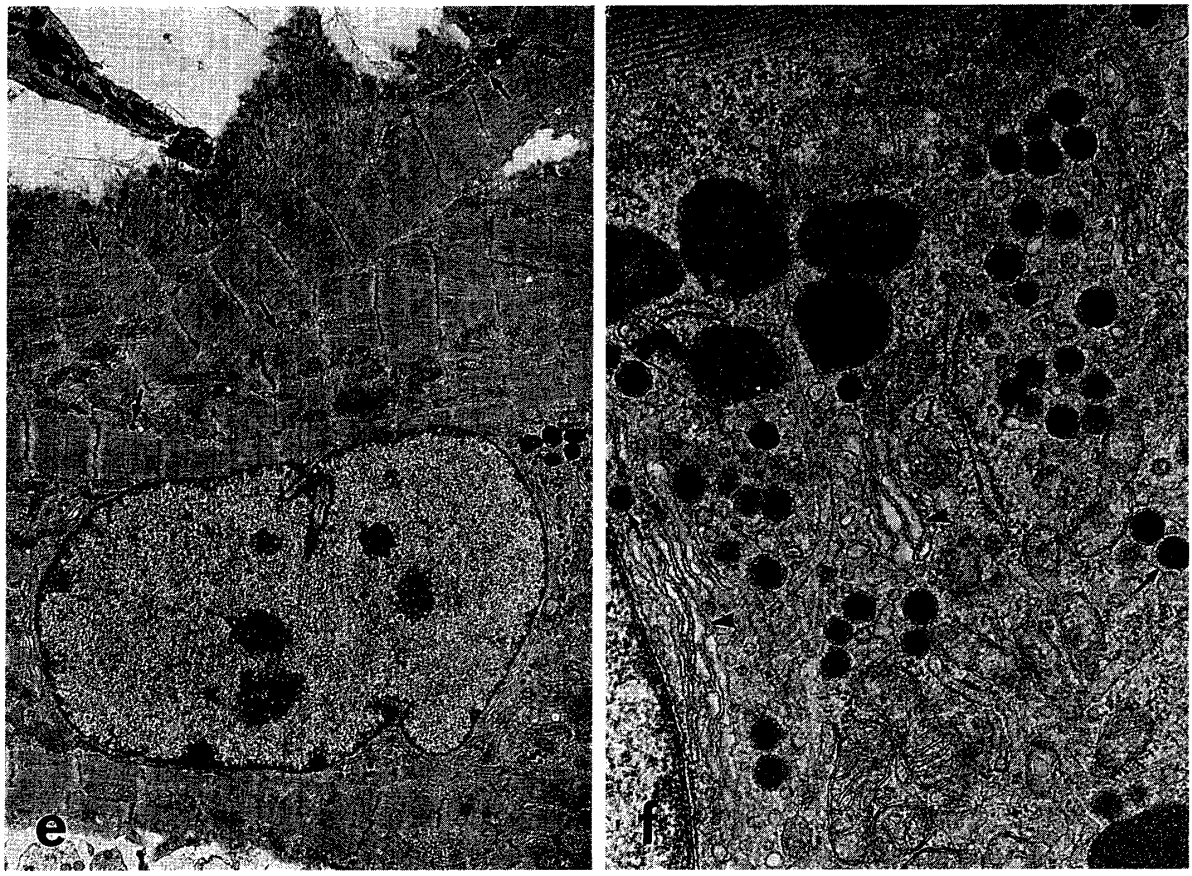


FIG. 4. Electron micrographs of cultured mouse ventricular myocardial cells in confluent sheets at 30 min after the onset of application of adrenochrome or epinephrine. (a) Mitochondrial fusion (arrows). 10^{-4} M adrenochrome. ×23,500. (b) Atrial specific granules (arrows) appeared. 10^{-4} M adrenochrome. ×4,700. (c) Almost all mitochondria became slightly swollen (arrows), and occasionally some revealed ischemic appearances (arrow head). 2×10^{-4} M adrenochrome. ×18,900. (d) Mitochondrial fusion (arrows) and giant mitochondrion (arrow head). 10^{-6} M epinephrine. ×18,900. (e) Atrial specific granules increased in number and appeared even among myofibrils (arrows). Thickness of myofibrils was increased, compared with figure 4b (where myofibrils were of normal size). 10^{-3} M epinephrine. ×3,900. (f) Many atrial specific granules (arrows) appeared in the perinuclear region and there was abundant Golgi apparatus (arrow heads). 10^{-3} M epinephrine. ×23,500.

former response was negative chronotropic one (Fig. 1a) but the latter positive one (Fig. 2a). Secondly, the dose inducing a necrotic response was also different between the two culture systems; single cells often revealed beat arrest in adrenochrome at a relatively low concentration, 10^{-5} M, and cell death at 10^{-4} M. But the cells in the sheets did not have such responses even at 2×10^{-4} M up to 2 hr after application (Fig. 3a) and had at a higher dose, namely 10^{-3} M, although mitochondrial swelling had already appeared 30 min after the onset of application of 2×10^{-4} M adrenochrome (Fig. 4c). In general, grouped cells may be more resistant to various attacks than single cells, probably due to intercellular cooperation by transporting various substances through gap junctions [6, 7], and it is possible that this mechanism can function in setting up the differential chronotropic responses of these culture systems.

Mitochondrial swelling was observed after application of 2×10^{-4} M adrenochrome (Fig. 4c), which was in agreement with the finding of Dhalla and his co-workers using perfused isolated hearts and intact hearts [23, 24, 29]. However, the dose of adrenochrome required to induce necrotic responses was different between the isolated or intact hearts and the present cultured myocardial

cells in sheets; in the former preparations intracellular edema, swelling of sarcoplasmic reticulum, contracted sarcomeres and dissolution of myofibrils were observed with application of adrenochrome at a 10^{-4} M order of concentration. However, these changes were not observed in the cultured myocardial cells at a dose of 2×10^{-4} M, and necrogenic responses were detected only at a higher dose, 10^{-3} M (Fig. 3a). We observed that LDH leakage from non-beating fibroblast-like cell clusters did not increase till 3 hr after application of 10^{-3} M adrenochrome (data not shown). This observation may indicate that cell membrane injury responsible for an increase of LDH leakage might be aggrevated by contractile movements. Since the contractile movement of the cultured myocardium in sheets should be less active than that of isolated or intact hearts, it is likely that the necrogenesis of adrenochrome might be less potent in cultured ventricular cells in sheets.

The *in vivo* production of a high concentration of adrenochrome, such as an order of 10^{-4} M, might not be predicted from the physiological serum peak concentration of 10^{-8} M of epinephrine [28] except in limited myocardial loci, such as subneuronal terminals which could be exposed to high concentrations of epinephrine. It is unlikely that adrenochrome plays an important role *in vivo* in inducing catecholamine cardiotoxicity, although there remains a possibility that even a low concentration of adrenochrome exhibits a synergic cardiotoxic action in the presence of an additional cell injury factor such as other drugs, oxygen lack, and so on.

Epinephrine induced positive chronotropic effects on both single ventricular cells (Fig. 1b) and cells in sheets (Fig. 2b). The drug failed to induce necrogenic responses even at 10^{-3} M, as confirmed by enzyme leakage (Fig. 3b) and ultrastructural studies (Figs. 4d-f). This differs from the results with myocardial enzyme leakage of Wheatley *et al.* [28] using perfused isolated hearts, when the perfusion pressure was high, and also differs from the ultrastructural results of Ferrans *et al.* [4], using intact hearts. In our experimental model, a hypoxic effect could not be supposed to take place, since myocardial cells were soaked in oxygen-rich media. This is different from isolated perfused hearts or intact hearts, which might be subjected to oxygen lack due to insufficient coronary circulation or to metabolic acceleration [9, 22].

Mitochondrial fusion was observed after application of either adrenochrome or epinephrine (Figs. 4a, d). This mitochondrial change seems to be a common characteristic of cultured myocardial cells subjected to ethanol attack, as previously reported [16]. The number of atrial specific granules increased in the vicinity of the nucleus and also slightly among myofibrils, after application of either adrenochrome or epinephrine (Figs. 4b, e, f). Some occasions are known in which atrial specific granules are produced in cultured ventricular myocardial cells [2, 10, 16]. The granules often appeared in hypertrophied [28] and volume-overloaded [13] rat ventricles *in vivo*. Both mitochondrial fusion and an increase in the granules may indicate that the drugs have had a common pathway for induction of the effects, as in the case of some metabolic alterations.

There is a difference in the ultrastructural effects of epinephrine and adrenochrome; epinephrine induced an increase in size of myofibrils, but adrenochrome did not. This may suggest that metabolic acceleratory action of epinephrine causes myocardial hypertrophy. Consequently, it is likely that epinephrine *per se* up to 10^{-3} M is not able to induce cardiotoxicity in cultured myocardial cells, since fatal damages to cell membrane and mitochondria have not been perceived in cells to which epinephrine has been applied.

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