Increased connexin43 gap junction protein in hamster cardiomyocytes during cold acclimatization and hibernation

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Abstract

Objective: The physiology of hibernation is characterized by dramatic reductions of heart rate, respiration, metabolism, blood pressure and body temperature and by resistance to ventricular fibrillation. Gap junctions in the heart provide low resistance pathways, facilitating electrical and metabolic coupling between cardiac muscle cells for coordinated action of the heart and tissue homeostasis. The conductance of these junctions, and therefore their function, is likely to be affected by the physiological changes that take place during hibernation. Our objective was to quantitate gap junction protein levels in cold acclimatization, hibernation and arousal. **Methods:** We have used specific antibodies to connexins 43 and 40, in combination with confocal microscopy, to quantitatively analyze the expression of connexin protein in hamster (*Mesocricetus auratus*) left ventricles in four animal groups: normal controls at euthermy, cold controls (cold-exposed animals that did not undergo hibernation), hibernating animals and animals aroused from hibernation for 2 h. **Results:** Connexin40 immunostaining was not detected in ventricular cardiomyocytes in any animal group but connexin43 was found in all groups. Connexin43 expression was significantly enhanced in hibernation and cold control ventricular cardiomyocytes. Total plaque area, numerical density and plaque size were higher in the cold controls and hibernating hamsters compared to normal controls and animals aroused from hibernation. **Conclusion:** It is possible that the increased size and number of connexin43 gap junction plaques in the cold controls may represent a compensatory response in order to maintain sufficient gap junction communication during physiological conditions that would reduce conductance. These changes may represent a mechanism by which the hamster avoids ventricular fibrillation during hibernation and arousal. © 2000 Elsevier Science B.V. All rights reserved.

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This article is referred to in the Editorial by T. Opthof and M.B. Rook (pages 6–8) in this issue.

1. Introduction

Hibernation of mammals is a complex adaptive phenomenon to facilitate survival during unfavourable environmental conditions, cold acclimatization and food shortage. Under these condition; organ functions and energy expense is depressed. The heart rate of hibernating rodents decreases from 350 beats/min to 2–4 beats/min [1]; respiration rate falls from 35 to 120 breaths/min to <5 breaths/min [2]; metabolism is reduced to 1–5% of the rate during normothermia [3]; body temperature reduces from 36 to 37°C to 5–10°C [2]; blood pressure declines and peripheral vascular resistance is increased [4].

Mammalian hibernators can periodically and spontaneously arouse from hibernation and elevate their body temperature to 37°C, an event requiring an intense thermogenic effort [5]. Arousal starts with an increase in heart rate, respiratory rate and oxygen consumption, followed by a rise in body temperature using only endogenously generated heat [6]. In the hamster, full arousal from

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hibernation takes approximately 2–3 h [7,8]. Both hibernation and arousal occur without pathological consequence.

Gap junctions in the heart provide low resistance intercellular channels to facilitate synchronous contraction of the myocardium. Connexins (Cx) are the major structural proteins of gap junctions and are commonly known either according to their MW or under a Greek nomenclature system (e.g. Cx43 or $\alpha 1$; Cx40 or $\alpha 5$). Cx43 is the principal connexin of mammalian heart and is found in large quantities in ventricles and atria [9,10]. Cx40 is abundant in the atrioventricular conductive system (His bundle, bundle branches and Purkinje fibres), atria and in the endothelium of many blood vessels [11–16]. Gap junctions are not static but undergo a continual process of formation and removal. The half-life of Cx43, for example, can be as short as 1.5–3.5 h, allowing significant potential for remodelling [17,18].

In the present study, we have investigated the immuno-expression of Cx43 and Cx40 in hamster left ventricle myocardium in hibernating animals, animals aroused from hibernation for 2 h normal controls and animals maintained in the cold that did not enter hibernation. We quantified the density and plaque sizes of Cx43 gap junction proteins in these groups.

2. Methods

2.1. Animals

Male golden hamsters (*Mesocricetus auratus*) 12 weeks old, and weighing 140–150 g, were used for the study. In these animals, hibernation can be induced by shortening the photoperiod and reducing the external ambient temperature. The investigation was performed in accordance with the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986, published by HMSO, London.

2.2. Induction of hibernation

Hamsters were initially placed in a Leec refrigerated incubator (model PL3) at a temperature of 20°C with a light: dark photoperiod of 8:16 h. The animals were kept 5 to a cage with food and water ad libitum. The temperature was gradually reduced by 1°C per day together with a reduction of 30 min of light per day until a temperature of 5°C and a photoperiod of 2:22 h was reached. The hamsters were then transferred to a cold room at a temperature of 5°C with 2 h of light per day. The hamsters were housed individually at this point and nesting material was supplied. Food and water were supplied ad libitum.

The hamsters remained in the cold room for 8–10 weeks before starting to hibernate. Once a hamster had gone into hibernation it was allowed to hibernate for 8 weeks before being killed. During this time the hamsters under went

'bouts' of hibernation, lasting about 3–4 days, with periods of alertness in between, lasting 1–2 days. An animal was sacrificed only after a minimum of 3 days into a hibernation bout. Those hamsters that failed to hibernate after the 8- to 10-week period, or subsequently (animals were checked daily), were termed cold-control animals. In addition age-matched (normal) controls which had not been exposed to a reduced temperature or photoperiod were also used. Hibernation was judged by the lack of response to a physical stimulus, namely the sprinkling of sawdust onto the back of a hamster (a sleeping hamster always awoke to this stimulus, whereas a hibernating animal would not). Those hamsters to be aroused from hibernation were brought into room temperature (22°C) and left for 2 h (these animals had been in deep hibernation for 3 days). Within this time the animal aroused fully, with an increase in heart rate, breathing rate and body temperature. All animals were weighed, and cheek pouch and rectal temperatures were taken immediately after death.

2.3. Tissue preparation

Six hamsters from each of the four experimental groups (normal controls, cold controls, hibernated and aroused) were killed by carbon dioxide asphyxiation. The outer wall of the left ventricle was quickly dissected out and embedded in OCT, precooled in isopentane slush then frozen in liquid nitrogen, and stored at −70°C until sectioning. Cryosections were cut at a thickness of 10 μm (Reichart cryostat) and mounted onto albumin-coated slides.

2.4. Antibodies

The immunolabelling studies utilized a commercially available polyclonal antibody to Cx43 (Zymed Laboratory, Inc, USA) and Cx40 antibody (kindly provided by Dr R.G. Gourdie). Polyclonal rabbit anti-Cx43 was raised against a peptide corresponding to a portion of the cytoplasmic tail (C-terminal). The anti-Cx40 antiserum was raised in rabbit against the peptide corresponding to residues 256–270 of the cytoplasmic C-terminal tail of rat Cx40 sequence [19].

2.5. Immunostaining

Immunofluorescent histochemistry of tissue sections used rabbit polyclonal antipeptide antisera against Cx43 and Cx40. In brief, tissue sections were fixed in acetone then incubated for 1 h at room temperature with primary antibodies: anti-Cx43, 1:500 dilution in phosphate buffer saline (PBS) containing 0.1 M L-lysine, 0.01% bovine serum albumin and 0.1% Triton-X100 (dilution buffer) and anti-Cx40, 1:800 dilution in the dilution buffer. After washing in PBS, the sections were incubated in the secondary antibodies, biotinylated anti-rabbit IgG (Amersham, UK), diluted 1:200 in dilution buffer for 30 mm.

After rinsing in PBS they were incubated in 1:200 Streptavidin-fluorosein isothiocyanate conjugate (Amersham) for 30 min. The sections were rinsed in PBS, and then mounted in Citifluor (London, UK). For controls, the same procedure was followed; however, (1) the primary antibody was substituted with dilution buffer, or (2) tissue was incubated with normal rabbit serum (Nordic) in place of primary antibody, or (3) tissue was incubated with primary antibody preabsorbed with Cx43 peptide (10 μg/ml with 1 μg/ml of primary antibody). Care was taken to ensure that dissecting, sectioning, and staining of tissues were done under identical conditions. Immunostained sections were examined blind on a Leica TCS 4D confocal microscope immediately after immunostaining. Optical images were stored digitally for subsequent analysis.

2.6. Techniques for quantification

The design of the analysis was as follows. From each of the four groups, the left ventricle longitudinal sections of six individual animals were used. Five randomly selected fields were optically sectioned in five 1-μm steps. Sections were scanned under identical parameters of imaging, pinholes, objective, filter and laser power. Levels were set according to procedures standardized to ensure that the image collected displayed a full range of grey level values from black (0 pixel intensity level) to peak white (255 pixel intensity level). Each image was signal-averaged (line average=8) during acquisition to reduce noise levels and improve image quality. The entire Z series was finally projected as a single composite image by superimposition. Quantification of Cx43 expression was performed according to Gourdie and colleagues [20]. The final image was thresholded to form a binary image for analysis by NIH image software 1.62.

Five optical fields×six animals from each group, were analyzed for: the mean gap junction plaque size (μm^2) (total area of junctions/total number of junctions); the area density (total area of junction/1000 μm^2) and numerical density (total number of plaques/1000 μm^2).

2.7. Statistical analysis

All data are expressed as the mean±standard error of mean (SEM) of 6 animals in each group. Analyses and tests were carried out using Minitab software (Minitab Inc. and StatView Pak). Statistical differences between the four groups were determined by analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered as significant. Statistical differences between the frequency distribution of plaque sizes for the four animal groups were determined using the Kolmogorov-Smirnov two-sample test (GB-Stat School Pak).

3. Results

3.1. Animals

The body weight of the animals and their cheek pouch and rectal temperatures are summarized in Table 1. The mean body weight of animals from the hibernating and aroused groups were significantly less than those from the cold control and normal control groups while the mean body weight of the cold controls was significantly less than the normal controls (Table 1). The cheek pouch and rectal temperatures were significantly lower ($P<0.05$; ANOVA) in the hibernating animals only (Table 1).

3.2. Cx43-Immunolabelling of left ventricle

In ventricular tissues of all animal groups, the anti-Cx43 antibody mainly stained at intercalated disk structures between cardiomyocytes as bright punctate staining with very little on the longitudinal cell border (Fig. 1). Cold controls and animals in hibernation exhibited higher labelling levels compared with the normal control and arousal groups (Fig. 1b and 1c compared to 1a and 1d, respectively). Immunostaining for Cx43 protein in heart blood vessels was not detected. Heart tissue sections incubated in anti-Cx43 antiserum preabsorbed with the parent peptide or normal rabbit serum showed no detectable fluorescence signal.

Histograms showing the results of quantitative analysis of Cx43 gap junction plaque area and numerical densities (per 1000 μm^2) and plaque size (μm^2) for the four animal groups are shown in Fig. 2. In normal controls ($n=6$), the area density was 4.03±0.93 μm^2/1000 μm^2, the numeri-

Table 1
Body weights, cheek pouch and rectal temperatures of hamsters from the 4 experimental groups expressed as mean±SEM[a]

	Normal control	Cold control	Hibernator (8 weeks)	Aroused (2 h)
Body weight (g)	165.4±7.2	136.8±4.6	104.1±5.2*	98.6±2.0*
Cheek pouch temperature (°C)	35.1±0.2	35.0±0.5	9.6±0.5**	34.6±0.4
Rectal temperature (°C)	32.2±0.5	32.1±0.4	9.8±0.5*	31.5±0.5

[a] Six animals in each group were used in this study.
* Significant difference from normal control and cold control, $P<0.05$.
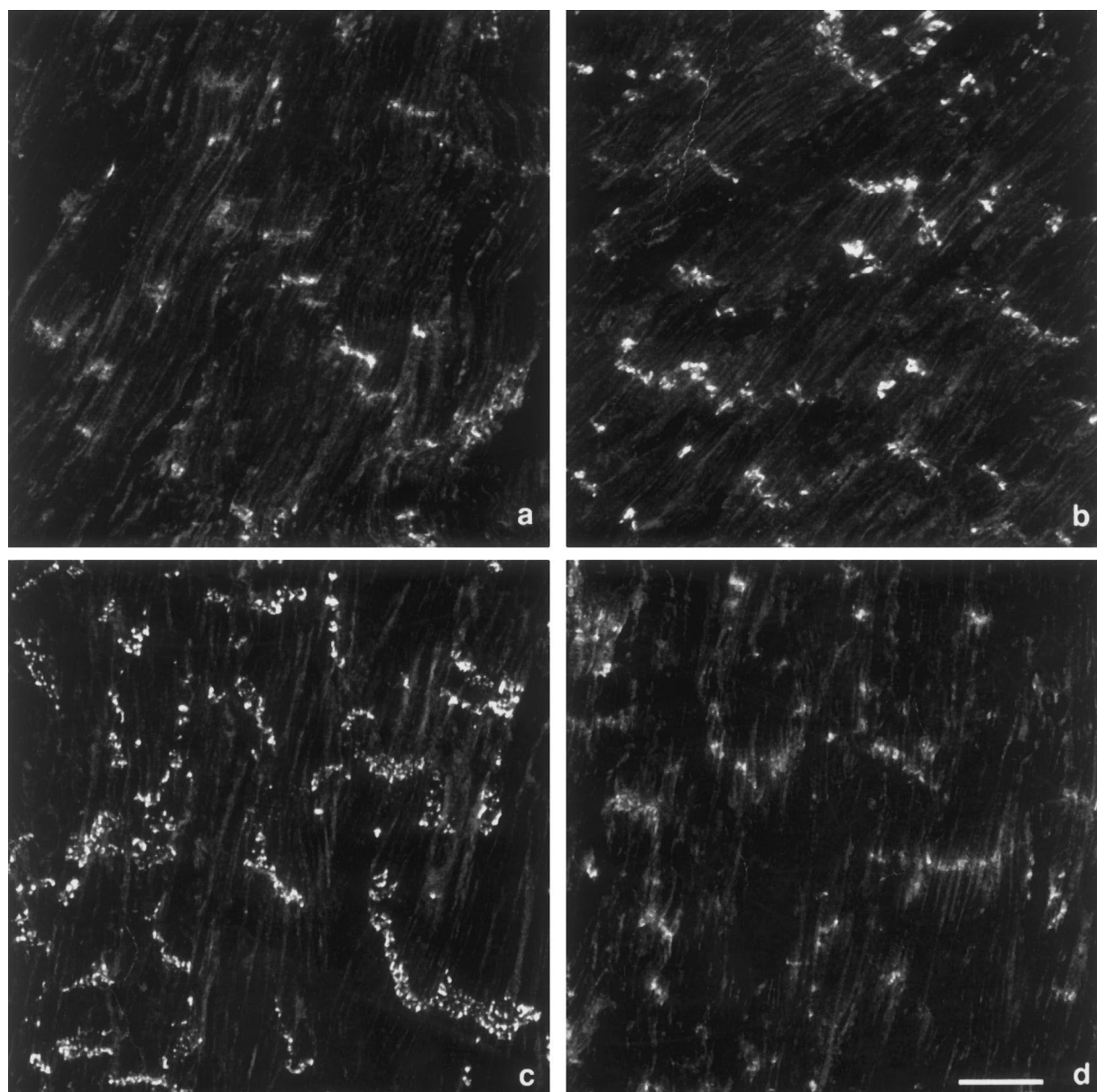** Significant difference from normal control and cold control, and arousal, $P<0.05$.

Fig. 1. Confocal fluorescent microscopy of anti-Cx43 immunostaining in left ventricle of normal control (a), cold control (b), hibernation (c), and aroused (d) hamsters. Projectional images were constructed from 5 optical sections. The lines of punctate staining represent gap junctions organized in the pattern of the intercalated disk between longitudinally oriented cardiomyocytes. Note higher intensity of the immunolabelled gap junctions in hibernation (c) and cold control (b). Note some labelling located on the lateral surface of the cells. Scale bar=10 μm.

cal density was 10.6 ± 1.73 plaques/1000 μm^2 and the mean plaque size was 0.35 ± 0.05 μm^2. Compared with the normal controls, there was a tendency for an increase in total plaque area and numerical density in the cold controls and a significant increase in plaque size: there were significant increases in all parameters in the hibernation group ($P<0.05$; ANOVA followed by Tukey's test). There were no significant differences between the cold control and hibernation groups. In the arousal group, area density, numerical density and plaque size were all significantly reduced compared to the hibernation group and were not significantly different from normal controls.

Frequency distributions of plaque sizes in the four groups of hamsters are shown in Fig. 3. Cx43 plaque sizes ranged mainly from 0.02 to 5 μm^2 whereas approximately 1% of the total population of plaques was larger than 5 μm^2 (not shown). The majority of plaques were less than 0.5 μm^2 (normal control, 79%; cold control, 74%; hibernation, 72% and arousal, 79%). Whilst the overall pattern of frequency distribution in all four groups appeared similar by eye on the scale shown, statistical analysis showed a significant difference between the normal controls and aroused groups compared with the cold control and hibernation groups ($P<0.05$; Kolmogorov-Smirnov two-
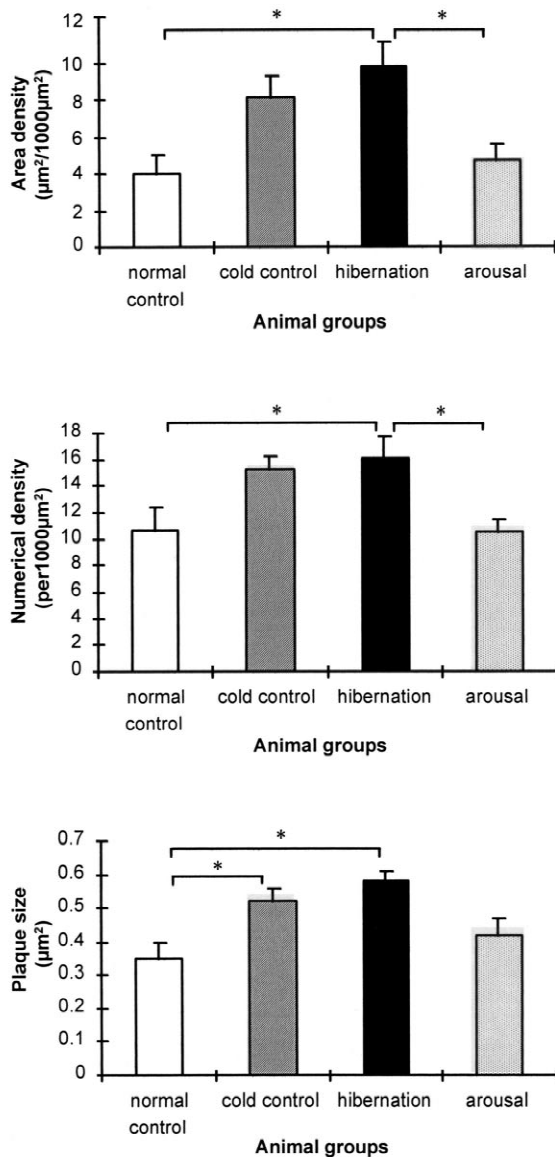
Fig. 2. Histograms showing results of quantification of Cx43 gap junction area and numerical densities and plaque size, expressed from the values obtained from projection images of five levels (each interval of 1 μm optical section) in five randomly examined areas for each individual animal tissue. The data are from the left ventricle of six animals from each group and expressed as mean±SEM. *, Significant difference between groups marked by horizontal bar $P<0.05$.

sample test) reflecting an increase of larger sized plaques in the latter groups as shown in be values for mean plaque sizes (Fig. 2).

3.3. Cx40-Immunolabelling of ventricle cardiomyocytes

Immunostaining for Cx40 was confined to the endothelium of blood vessels, with no staining in the vascular smooth muscle or cardiomyocytes. There was no expression of Cx40 detected in ventricular cardiomyocytes in any of the experimental groups.

4. Discussion

Cx43 gap junctions in normal hamster left ventricle were punctate, precisely demarcating intercalated disks, consistent with previous reports of Cx43 labelling of mammalian heart [20–22]. Quantitative analysis of Cx43 gap junction plaque size of the normal controls was very similar to those for hamster ventricle reported by Gourdie et al. [20]. This confirms that our staining is real and at normal levels in control animals. Any differences detected in connexin expression can therefore be interpreted as due to the physiological changes associated with the experimental conditions.

The main finding of the present study was an increase in ventricular cardiomyocyte Cx43 immunolabel and in hamsters maintained in the cold with a short photoperiod and in hamsters during hibernation with a reduction to normal control levels within 2 h of arousal to euthermy. The quantitative analysis showed significantly higher Cx43 gap junction expression in terms of the number of plaques and in the plaque area during hibernation compared with normal controls. In cold controls and hibernation the Cx48 gap junction plaque size was significantly higher than in normal controls. Differences in the frequency distribution of gap junction plaque size confirmed an increase in larger plaques in cold controls and hibernators. There were no significant differences in Cx43 expression between cold controls and hibernators. In aroused animals, there was a marked return of all parameters of Cx43 expression to normal control values: there were no significant differences between aroused and normal control groups.

Our finding that Cx40 was not present in the ventricular cardiomyocytes of any of the animal groups examined indicates that there is no new expression of this connexin in the myocardium during cold exposure, hibernation or arousal.

Our data indicate that, in the hamster, chronic periods in the cold and/or reduced daylight, initiate the increase in Cx43-immunoexpression in ventricular cardiomyocytes similar to levels noted during hibernation. This is perhaps surprising since the physiological responses to chronic exposure to the cold are opposite to those occurring during hibernation. During cold exposure there is increased sympathetic activity leading to stimulation of thyroid hormone release and an increase in body metabolism [23]. There is rising thermogenesis, mainly from brown adipose tissue, to produce heat to maintain core temperature [24,25]. Heart rate rises requiring a reduction in the duration of action potentials in atrial and ventricular fibres [26] and increased oxygen consumption increases stroke volume to increase cardiac output [25,27]. Rats exposed to the cold for 1–3 weeks become hypertensive with tachycardia ant cardiac hypertrophy [28]. As there is a similar increase in Cx43 levels in the myocardium from the cold control and hibernation groups, the factors involved in regulating this increase are unlikely to be related to core
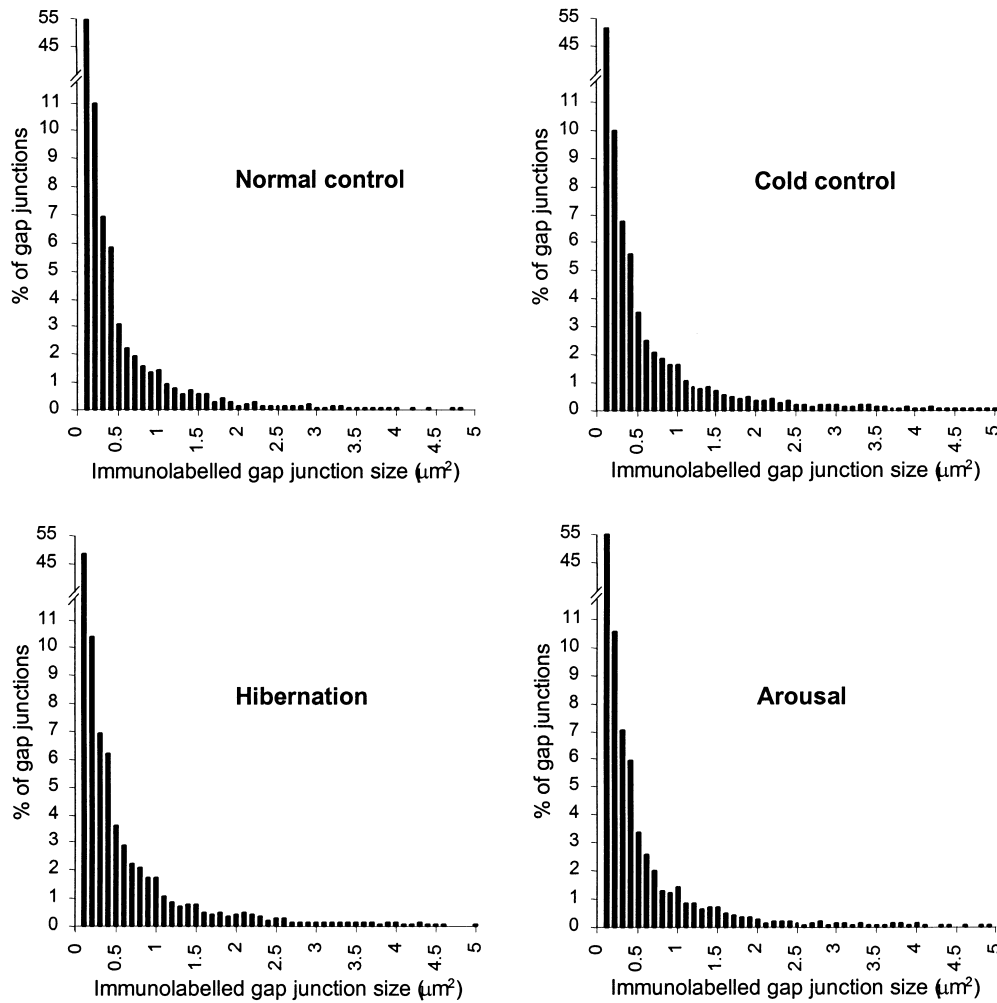
Fig. 3. Histograms showing frequency distributions of Cx43 immunopositive gap junction plaque sizes, as measured by fluorescent spot in pixels (1 pixel=0.024 μm^2), in left ventricular myocardium of four experimental hamster groups: normal control; cold control; hibernation; arousal. Bars represent the actual percentage of gap junction plaques of increasing size. A few immunostained junctions above 5 μm^2 were detected (not shown) accounting for less than 1% of the total population of gap junctions. Larger plaques were more frequent in the cold control and hibernation groups compared to the normal control and aroused groups ($P<0.05$).

body temperature, metabolism or heart rate as these are all dramatically reduced during hibernation compared to the cold controls.

An increase in immunodetectable levels of Cx43 may reflect increased Cx43 mRNA expression. Alternatively, or in addition to this, there may be reduced degradation of Cx by proteasomal and lysosomal enzymes. Indeed as heat shock proteins are increased in cold stress and there are indications that these, specifically HSP70, protect against Cx43 degradation [17,29] this could be a major mechanism underlying increased gap junctional plaque size in the cold control and hibernation groups. Increased sympathetic activity, as occurs during cold exposure, may also influence Cx43 levels via humorally mediated β-adrenoceptor activation. There are, however, no sympathetic nerves to the ventricular myocardium in hamsters; sympathetic terminals are restricted to the conductive fibres and myocardial blood vessels [30]. This is in contrast to non-

hibernating mammals where the ventricular myocardium is richly innervated by sympathetic nerves [30]. It will be important to know whether the increased Cx43 immunoexpression is specific to hibernators or whether non-hibernating mammals also show this change on cold exposure.

Whilst our study does not address the functional state of gap junctions formed [reviewed in [31–33]], it may be anticipated that during hibernation, low body temperature is a principal physiological factor affecting ventricular myocyte gap junction communication by decreasing the frequency of channel opening [34,35]. Furthermore, there is a decrease of cAMP formation in ventricles during hibernation which may also reduce cell-to-cell communication [36]. Other factors which influence gap junction communication, for example $[Ca^{2+}]$ and pH, are well regulated during hibernation [37–39]. Thus, although ventricular Cx43 levels are increased following chronic cold exposure and during hibernation, the cell-to-cell

myocardial communication through these gap junction channels is likely to be diminished during hibernation.

Non-hibernating mammals are susceptible to ventricular fibrillation as core temperature decreases below 20°C [40,41]. Several characteristics of the hibernator heart confer resistance to ventricular fibrillation including physicochemical properties of lipids, avoidance of Ca^{2+} overload, lack of sympathetic innervation to the ventricular myocardium and inability to generate long QT intervals [41]. Inappropriate myocardial intercellular coupling has been implicated in arrhythmogenesis [10]. One explanation for the cold exposure/reduced daylight-induced changes in gap junction plaque density and size in the heart of the hamster may be to prepare the animal for ventricular fibrillation tolerance as it enters hibernation.

On arousal from the hibernating state, the reduction of Cx43 gap junctional protein content and plaque size to euthermic controls levels within 2 h from the beginning of arousal is quite remarkable. A similar dramatic change in gap junctions has previously been reported in the pregnant myometrium with an increase in gap junctions with the onset of labour and rapid reversal following delivery, corresponding to transcriptional regulation of Cx43 mRNA expression [42,43]. Our data demonstrate that rapid changes in Cx43 gap junctional protein can also occur in the hamster heart to accommodate the physiology of hibernation and arousal.

Acknowledgements

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