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Thyroid status and β-agonistic effects on cytosolic calcium concentrations in single rat cardiac myocytes activated by electrical stimulation or high-K^+ depolarization

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The effects of the thyroid status on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cardiomyocytes were studied at rest and during contraction. The mean resting $[Ca^{2+}]_i$ increased significantly from the hypothyroid (45 ± 4 nM) through the euthyroid (69 ± 12 nM) to the hyperthyroid condition (80 ± 11 nM) at extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) up to 2.5 mM. At $[Ca^{2+}]_o$ above 2.5 mM the differences in $[Ca^{2+}]_i$ between the groups became less. The amplitude of the Ca^{2+} transients became higher in all groups with increasing $[Ca^{2+}]_o$ (1, 2.5 and 5 mM), and was highest at all $[Ca^{2+}]_o$ in hyperthyroid myocytes. The β-agonist isoprenaline elevated peak $[Ca^{2+}]_i$ during contraction and increased the rate of the decay of the Ca^{2+} transients to a greater extent in hypothyroid myocytes than in hyperthyroid myocytes. Depolarization with high $[K^+]_o$ induced a large but transient $[Ca^{2+}]_i$ overshoot in hypothyroid myocytes, but not in hyperthyroid myocytes, before a new elevated steady-state $[Ca^{2+}]_i$ was reached, which was not different between the groups. When isoprenaline was added to K^+_o-depolarized myocytes after a steady state was reached, a significantly larger extra increase in $[Ca^{2+}]_i$ was measured in the hypothyroid group (28%) compared with the hyperthyroid group (8%). It is concluded that in cardiac tissue exposed to increasing amounts of thyroid hormones (1) $[Ca^{2+}]_i$ increases at rest and during contraction in cardiomyocytes and (2) interventions which favour Ca^{2+} entry into the cytosol ($[Ca^{2+}]_o$ elevation, high $[K^+]_o$, β-agonists) tend to have less impact on Ca^{2+} homoeostasis.

INTRODUCTION

When a cardiac cell is stimulated, Ca^{2+} enters the cytosol mainly by two pathways. The predominant one is Ca^{2+} released from the sarcoplasmic reticulum (SR), and to a lesser extent there is Ca^{2+} influx from outside the cell through slow Ca^{2+} channels [1]. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) rises and binding of Ca^{2+} to the contractile filaments results in contraction. Next, Ca^{2+} is removed from the cytosol mainly by Ca^{2+} uptake into the SR and Ca^{2+} transport out of the cell by sarcolemmal Ca^{2+} pumps and Na^+/Ca^{2+} exchange. It is clear that hormones or other factors which stimulate one or more of these Ca^{2+}-transfer mechanisms might influence $[Ca^{2+}]_i$ and thereby contractility and metabolic activity of the cell. It is well known that thyroid hormones stimulate Ca^{2+} transport into the SR by increasing the number of Ca^{2+} pumps and the amount of SR [2,3]. There is also evidence that Ca^{2+} influx through slow Ca^{2+} channels is stimulated by thyroid hormone [4], as is Ca^{2+} removal from the cytosol by stimulation of the Ca^{2+}-ATPase in the sarcolemma [5]. The stimulation of these Ca^{2+}-transfer processes results in a faster contraction/relaxation cycle at a higher energy cost. A similar stimulatory action, but transient and on a shorter time-scale, is effected by β-agonists such as isoprenaline (isoproterenol). Ca^{2+} influx from outside the cell is stimulated by phosphorylation of the slow Ca^{2+} channels [6], and Ca^{2+} uptake into the SR by phosphorylation of a regulatory protein of SR Ca^{2+}-ATPase called phospholamban [7]. In view of the stimulatory effects of both compounds on Ca^{2+} transport, we have been interested for some time in their separate and combined effects on Ca^{2+} homoeostasis in cardiac cells [3,8].

Although in a previous study with myocyte suspensions we showed that the mean resting $[Ca^{2+}]_i$ was higher in hyperthyroid than in euthyroid myocytes, the differences were not significant. Quantification of $[Ca^{2+}]_i$ in hypothyroid myocytes was complicated by the occurrence of variable proportions (5–40%) of metabolically very active viable rounded myocytes with high $[Ca^{2+}]_i$ levels [8]. Neither was quantification of $[Ca^{2+}]_i$ possible during contraction, since no exact knowledge of the proportion of contracting myocytes in the suspension could be obtained. Heterogeneity therefore complicates interpretation of data obtained on large populations of cells. These difficulties can be mostly eliminated by using single-cell fluorescence microscopy. By using this technique we now report on the changes in $[Ca^{2+}]_i$ occurring during rest and electrical stimulation or high-$[K^+]_o$ depolarization in single myocytes of hypothyroid, euthyroid and hyperthyroid rats. These experiments are supplemented with observations on the effects of the β-agonist isoprenaline under the above-mentioned conditions. The results show that, on average, higher cytosolic $[Ca^{2+}]_i$ levels in hyperthyroid myocytes are accompanied by a decreased responsiveness to interventions which normally elevate $[Ca^{2+}]_i$ levels, and that the opposite is found in the hypothyroid condition.

MATERIALS AND METHODS

Animals

Male rats of the Wistar strain were used in the experiments. To induce hypothyroidism, the animals were maintained on a low-iodine diet (Hope Farms, Linschoten, The Netherlands) with 2% (w/v) $KClO_4$ in their drinking water for 6 weeks [9]. Hyperthyroidism was induced by daily subcutaneous injections of 20 μg of 3,3',5-tri-iodo-L-thyronine/100 g body wt. for 7 or 8 days [9]. The animals in all experimental groups were weight-matched (250–280 g).

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration (similarly for K^+); $RC_{0.5}$, time to 50% decay of peak $[Ca^{2+}]_i$; SR, sarcoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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Myocyte isolation and fura-2 loading procedure

Cardiac myocytes were isolated as described in [8]. This method yields 60–70 % Ca^{2+}-tolerant myocytes. After the isolation and washing procedure, the suspension was adjusted to the desired cell density (16 mg wet wt./ml) and kept in Joklik-Modified minimal essential medium (MEM) supplied with 2 % (w/v) BSA and 1 mM-$CaCl_2$ under constant shaking and gassing with O_2/CO_2 (19:1). From this stock solution samples were taken for the fluorescence measurements. The cells were loaded for 15 min at 37 °C with fura-2/AM (0.08–0.6 μM) by adding 0.5 ml of the stock suspension to 1.5 ml of Joklik-MEM with 2 % BSA and 1 mM-$CaCl_2$, to which was added the desired concentration of fura-2/AM. Next, the loaded suspension was washed twice by centrifuging at 30 *g* for 20 s. The pellet was resuspended in 1.5 ml of oxygenated KH buffer, which contained (mM) NaCl (118), $NaHCO_3$ (12), KCl (2.6), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (1, 2.5 or 5), glucose (11) and BSA (0.2 %), at pH 7.2. This suspension contained approx. 3 mg of fura-2-loaded cells/ml. The intracellular fura-2 concentration was calculated as described previously [8].

Fluorescence measurements

A dilute suspension of cells that had been loaded with fura-2 was placed on a temperature-controlled coverslip chamber [10] on the stage of an inverted microscope, and the cells were allowed to settle. The cells were continually superfused with buffer solution at a rate of 3 μl/s at 37 °C. The cells were made to contract by applying external field stimulation (15 V pulses, 1.5 ms duration, 0.15 Hz). In each experiment at least four different myocytes were studied, and the values obtained were averaged.

The experimental set-up consisted of a modified Zeiss IM inverted microscope suitable for dual-wavelength excitation and measurement of fluorescent emission. A high-pressure mercury discharge lamp was used as excitation light source. Excitation wavelength was set by a filter-wheel (maximum revolution speed 250 rev./s) containing two interference filters, one that passes 340 nm light and other that passes 380 nm light. The excitation light that passed these filters was then diverted through the objective (Nikon UVF 40×) to the cell by a dichroic mirror (> 395 nm). The fluorescence light passed through an emission filter (510 nm) and was directed to the photocathode of the photomultiplier tube. The photomultiplier was coupled to an Analog-Digital (A-D) converter interfaced to a personal computer (Olivetti M24). A-D-converted signals were sampled and stored on hard disk for off-line analysis.

Calibration procedure

We applied intracellular calibration essentially as described by Li *et al.* [11]. To raise $[Ca^{2+}]_i$ for the measurement of maximal fura-2 fluorescence at 340 and 380 nm excitation, we used buffer without glucose, containing ionomycin (10 μM), KCN (2 mM) and FCCP (2 μM). To deplete $[Ca^{2+}]_i$ for the measurement of minimal fura-2 fluorescence, we used buffer without Ca^{2+}, to which was added 10 mM-EGTA and 10 μM-ionomycin. The values were corrected for autofluorescence at the two excitation wavelengths. An example of a calibration curve is shown in Fig. 1.

The $[Ca^{2+}]$ was calculated with a computer program (ANAFLU) from the ratio (R) of the fluorescence of the two wavelengths of excitation (340 and 380 nm) by using the equation:

$$[Ca^{2+}] = K_d \times \frac{R_{380}^{340} - R_{380\,min.}^{340}}{R_{380\,max.}^{340} - R_{380}^{340}} \times \frac{F_{min.}^{380}}{F_{max.}^{380}}$$

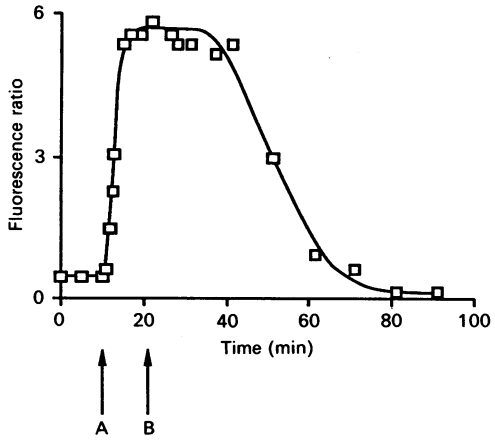

Fig. 1. Intracellular calibration ($R_{max.}$, $R_{min.}$) of a single cardiomyocyte

Superfusion of myocytes took place with standard medium containing 1 mM-Ca^{2+}. After 10 min (A), medium was switched to standard medium which contained ionomycin (10 μM), FCCP (2 μM) and KCN (2 mM) to promote Ca^{2+} entry into the cell and to deplete the cell of ATP. After attainment of the $R_{max.}$ value, medium was switched for a second time (B) to medium without added Ca^{2+} but with 10 mM-EGTA added, and $R_{min.}$ was determined.

$R_{min.}$ and $R_{max.}$ are the values of R obtained under conditions without Ca^{2+} and with saturating $[Ca^{2+}]$. $F_{min.}^{380}/F_{max.}^{380}$ is the ratio of the 380 nm fluorescence signals of fura-2 without Ca^{2+} and that with saturating $[Ca^{2+}]$. The K_d (214 nM) was experimentally determined as described by Grynkiewicz *et al.* [12].

The values obtained thus were (means ± S.D.): $R_{min.}$, 0.18 ± 0.02 (n = 20); $R_{max.}$, 3.9 ± 0.9 (n = 22); $F_{min.}^{380}/F_{max.}^{380}$ = 5.2.

Materials

The following chemicals were used: tri-iodo-L-thyronine, FCCP, KCN, (−)-isoprenaline (+)-bitartrate (Sigma Chemical Co., St. Louis, MO, U.S.A.); fura-2/AM, fura-2 (Molecular Probes, Eugene, OR, U.S.A.); ionomycin (Calbiochem., San Diego, CA, U.S.A.); Joklik MEM (Gibco, Amstelstad, Zwanenburg, The Netherlands); collagenase, hyaluronidase (Boehringer, Mannheim, Germany).

Statistical analysis

Statistical significance of differences between means was mostly estimated by using Student's t test for paired and unpaired analysis, but in some instances by two-way analysis of variance (ANOVA).

RESULTS

Fura-2 loading

In a previous study with myocyte suspensions, the lowest intracellular fura-2 concentration which enabled us to perform reliable $[Ca^{2+}]_i$ measurements was 70 μM [8]. At this loading level we still found significant buffering of the Ca^{2+} transients by fura-2. Our present epifluorescence equipment permitted measurements on single cells with fura-2 loadings as low as 20 μM. Table 1 shows that, at [fura-2] of 40 μM or lower, peak $[Ca^{2+}]_i$ during contraction and the half-time of decay of the Ca^{2+} transient were no longer influenced by the presence of the dye. In further experiments, cell suspensions were exposed to 0.15 μM-fura-2/AM, which resulted in an intracellular [fura-2] of 35 μM after 15 min of incubation.

Table 1. Effect of intracellular fura-2 on Ca^{2+} transients in single cardiac myocytes

Rat cardiomyocytes were loaded with 0.08, 0.15 or 0.6 μM fura-2/AM, which corresponded to intracellular fura-2 concns. ($[fura\text{-}2]_i$) of 20, 40 or 190 μM. These latter values were estimated as described in [8]. The cells were transferred to the cell chamber on the platform of the fluorescence microscope. Cells were superfused with medium containing 1 mM-Ca^{2+} and electrically stimulated at a frequency of 0.15 Hz. Only one myocyte was studied at a time. In each experiment the peak $[Ca^{2+}]_i$ and the time to half decay of the Ca^{2+} transient ($RC_{0.5}$) were calculated from averaged Ca^{2+} transients. All values are means ± S.E.M.: *$2P < 0.05$ versus 40 μM, by Student's t test.

Fura-2/AM load (μM)	$[Fura\text{-}2]_i$ (μM)	Peak $[Ca^{2+}]_i$ (nM)	$RC_{0.5}$ (ms)
0.08	20	175 ± 16 (4)	107 ± 4 (4)
0.15	40	201 ± 16 (4)	106 ± 5 (4)
0.60	190	130 ± 12 (3)*	205 ± 39 (3)*

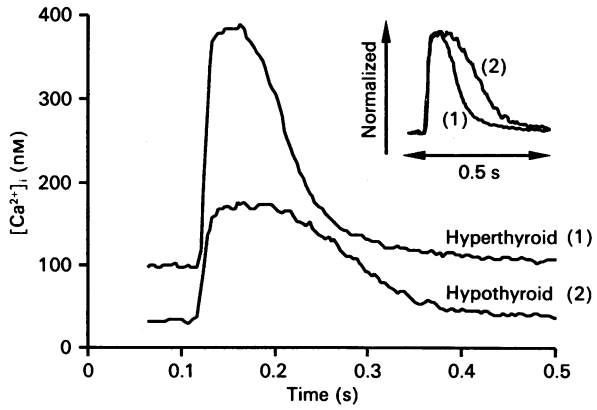

Fig. 2. Ca^{2+} transient from a hypothyroid and a hyperthyroid myocyte

An example is shown of a Ca^{2+} transient from a hypothyroid and a hyperthyroid myocyte at 2.5 mM-Ca^{2+}. The cells were loaded with fura-2 ($\leq$ 40 μM) and stimulated at a frequency of 0.15 Hz at 37 °C. The inset shows the normalized Ca^{2+} transients.

Table 2. Thyroid state and resting $[Ca^{2+}]_i$

Fura-2 (< 40 μM)-loaded myocytes from different thyroid states were superfused with medium of different $[Ca^{2+}]_o$ levels (1, 2.5, 5 mM) at 37 °C and electrically stimulated at a frequency of 0.15 Hz. The values represent the basal $[Ca^{2+}]_i$ levels (resting or diastolic $[Ca^{2+}]_i$) measured in the interval between two contractions. In each experiment at least five single myocytes were studied, and the values were averaged. Values represent means ± S.E.M. for four experiments. ANOVA of resting $[Ca^{2+}]_i$ values of the different groups at 2.5 mM-Ca^{2+}_o gave $2P \leq 0.05$; *$2P < 0.05$ versus hypothyroid, and †$2P < 0.05$ versus 1 mM-Ca^{2+}, by Student's t test.

$[Ca^{2+}]_o$ (mM)	Diastolic $[Ca^{2+}]_i$ (nM)		
	Hypothyroid	Euthyroid	Hyperthyroid
1	39 ± 8		64 ± 5*
2.5	45 ± 4	69 ± 12	80 ± 11*
5	65 ± 6†		79 ± 11

Table 3. Ca^{2+} transients during contraction

Fura-2 (< 40 μM)-loaded myocytes of different thyroid states were superfused with media of different $[Ca^{2+}]_o$ values (1, 2.5, 5 mM) at 37 °C. The cells were electrically stimulated at a frequency of 0.15 Hz. The upper part of the Table shows the peak $[Ca^{2+}]_i$ levels reached during contraction, and the lower part shows the half-time for the decay of the Ca^{2+} transient ($RC_{0.5}$). For other experimental details see Table 2. Values represent means ± S.E.M. for four experiments. ANOVA of the peak $[Ca^{2+}]_i$ values of the different groups at 2.5 mM-Ca^{2+}_o gave $P \leq 0.25$; †$2P < 0.05$ versus hypothyroid or euthyroid, and *$2P < 0.05$ versus 1 mM-Ca^{2+}, by Student's t test.

$[Ca^{2+}]_o$ (mM)	Maximum $[Ca^{2+}]_i$ (nM)		
	Hypothyroid	Euthyroid	Hyperthyroid
1	175 ± 21		249 ± 21†
2.5	270 ± 37	246 ± 31	329 ± 27†
5	286 ± 28*		391 ± 17*†
	$RC_{0.5}$ (ms)		
	Hypothyroid	Euthyroid	Hyperthyroid
1	154 ± 5		42 ± 8†
2.5	120 ± 9*	89 ± 6	59 ± 8†
5	124 ± 10*		62 ± 7†

$[Ca^{2+}]_i$ at rest

Table 2 shows the diastolic (resting) $[Ca^{2+}]_i$ in myocytes of different thyroid states at several extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$). A $[Ca^{2+}]_o$ of 1 mM corresponds to the free Ca^{2+} concentration in blood (total $[Ca^{2+}]_o$ 2.5 mM).

The results show that at 1 mM- and 2.5 mM-Ca^{2+}_o resting $[Ca^{2+}]_i$ is significantly higher in hyperthyroid myocytes than in hypothyroid myocytes. In agreement with our previous study with myocyte suspensions [8], we found lower mean $[Ca^{2+}]_i$ values in euthyroid myocytes than in hyperthyroid myocytes, but the difference was not significant, owing to the relatively large scatter of the data. However, taken together the results indicate that at physiological $[Ca^{2+}]_o$ a gradual rise in resting $[Ca^{2+}]_i$ occurs with increasing levels of circulating thyroid hormone. Resting $[Ca^{2+}]_i$ rose significantly in hypothyroid myocytes when $[Ca^{2+}]_o$ was increased from 1 to 5 mM, but not so in hyperthyroid myocytes.

$[Ca^{2+}]_i$ during electrical stimulation

An example of a Ca^{2+} transient from an electrically stimulated hypothyroid and hyperthyroid myocyte in buffer with 2.5 mM-Ca^{2+} is shown in Fig. 2.

Apart from the higher resting $[Ca^{2+}]_i$ in the hyperthyroid myocyte, the rise in $[Ca^{2+}]_i$ was also higher in the hyperthyroid condition. Furthermore, the decay time of the Ca^{2+} transient was much shorter in the hyperthyroid myocyte, which is once more illustrated in the inset in Fig. 2, where the Ca^{2+} transients were normalized. The results of experiments with electrically stimulated myocytes are summarized in Table 3, which shows that at all $[Ca^{2+}]_o$ levels the peak $[Ca^{2+}]_i$ values attained during contraction were higher in the hyperthyroid group than in the hypo- or eu-thyroid group. Furthermore, the peak $[Ca^{2+}]_i$ increased at rising $[Ca^{2+}]_o$; also in the hyperthyroid group, in spite of the higher $[Ca^{2+}]_i$ concentrations at rest. Both groups showed a 60% increase in peak $[Ca^{2+}]_i$ with a rise from 1 to

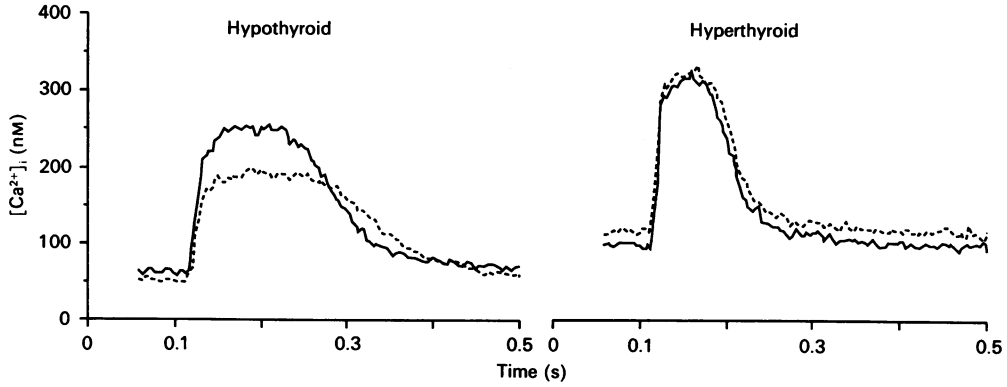

Fig. 3. Effect of isoprenaline on Ca^{2+} transients

An example is shown of Ca^{2+} transients from hypothyroid and hyperthyroid myocytes in the absence (----) and presence (——) of isoprenaline (1 μM). Other experimental conditions were as described in the legend to Fig. 2.

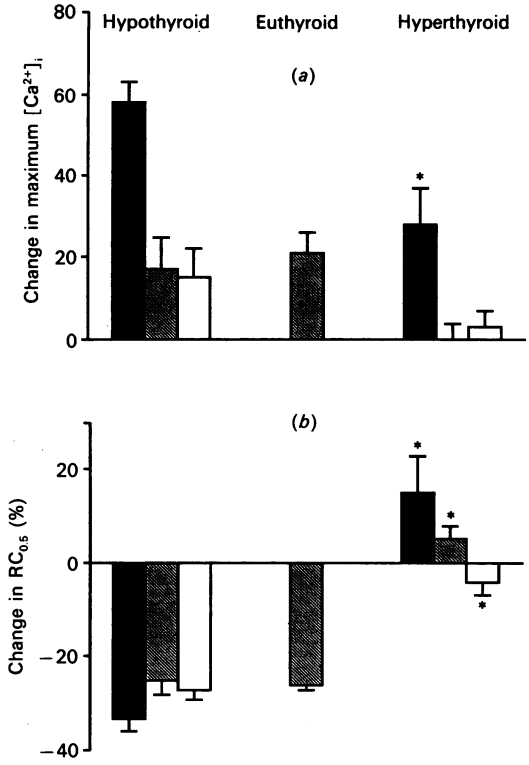

Fig. 4. Effect of isoprenaline on Ca^{2+}-transient parameters

Myocytes from different thyroid states were electrically stimulated at 0.15 Hz. Panel (*a*) shows the percentage change in maximal $[Ca^{2+}]_i$ at different $[Ca^{2+}]_o$ [(■) 1, (▩) 2.5, (□) 5 mM] in the presence of isoprenaline (1 μM) compared with the control values, i.e. the value before isoprenaline activation (100%). Panel (*b*) shows the percentage change in the half-time for decay of the Ca^{2+} transient $RC_{0.5}$ in the presence of isoprenaline (1 μM) compared with control values. Bars represent means ± S.E.M. ($n = 4$); *$2P < 0.05$ versus hypothyroid.

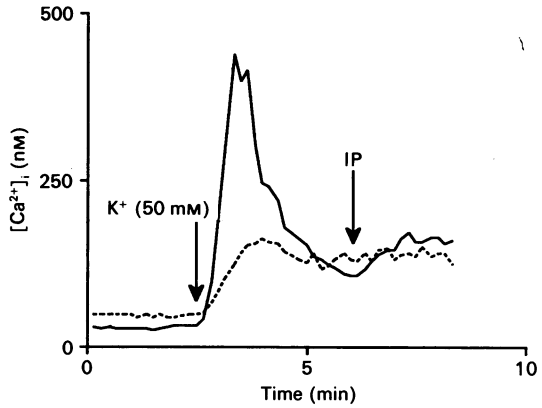

Fig. 5. Effect of high $[K^+]_o$ on cytosolic Ca^{2+}

An example is shown of single fura-2-loaded resting (——) hypo- and (----) hyper-thyroid myocytes exposed to medium with 50 mM-K^+_o at 37 °C. After about 6 min, when a new steady-state $[Ca^{2+}]_i$ was established, medium was switched to medium containing 1 μM-isoprenaline (IP).

5 mM-Ca^{2+}_o. This indicates that Ca^{2+} homoeostasis is equally affected in both groups by variation of $[Ca^{2+}]_o$ during electrical stimulation. The lower part of Table 3 summarizes the results on the half-time of decay ($RC_{0.5}$) of the Ca^{2+} signal. At all $[Ca^{2+}]_o$ (1, 2.5, 5 mM) the $RC_{0.5}$ was considerably longer in the hypothyroid group than in the hyperthyroid group. The differences became less with rising $[Ca^{2+}]_o$ (1 mM, 350%; 5 mM, 100%).

Effect of the β-agonist isoprenaline

The inotropic effect of isoprenaline on the heart is well known, and by using the bioluminescent Ca^{2+} indicator aequorin [13] it has been shown to be accompanied by an extra rise in $[Ca^{2+}]_i$ during contraction. This is thought to be the net effect of decreasing affinity of troponin for Ca^{2+} and of stimulation by isoprenaline of several Ca^{2+}-transfer pathways, such as Ca^{2+} influx through slow Ca^{2+} channels and both Ca^{2+} influx and efflux through the SR. We were interested in the effect of the thyroid status on isoprenaline-induced $[Ca^{2+}]_i$ changes, since we showed in a previous study that hyperthyroidism virtually eliminates the effect of isoprenaline on Ca^{2+} uptake into the SR [3]. An example of the effect of isoprenaline (1 μM) on Ca^{2+} transients of a contracting hypothyroid and hyperthyroid myocyte is presented in Fig. 3. It shows that isoprenaline increased maximum $[Ca^{2+}]_i$ in the hypothyroid myocyte, but not in the hyperthyroid myocyte. Equally, the decay of the Ca^{2+} transient was not affected by isoprenaline in the hyperthyroid myocyte, but was accelerated by isoprenaline in the hypothyroid myocyte. Fig. 4 summarizes the results.

Fig. 4(*a*) shows first that the percentage change in maximal $[Ca^{2+}]_i$ induced by isoprenaline became less in both the hypo- and

Table 4. Thyroid state and high-[K^+]$_o$ (50 mM) depolarization

Myocytes of hypo- and hyper-thyroid-rat hearts were loaded with fura-2 (< 40 μM) and exposed to 50 mM-K^+_o medium after equilibration in standard medium with 5 mM-K^+_o. These myocytes were not electrically stimulated, and the resting [Ca^{2+}]$_i$ value before high-[K^+]$_o$ depolarization was 27±2 (n = 5) and 43±10 (n = 4) in the hypothyroid or hyperthyroid group respectively. A switch to 50 mM-K^+_o medium with isoprenaline (1 μM) took place after about 6 min, when [Ca^{2+}]$_i$ had reached a new steady state during high-[K^+]$_o$ exposure. Maximal [Ca^{2+}]$_i$ represents the peak Ca^{2+} level reached during high-[K^+]$_o$ exposure. In each experiment at least five single myocytes were studied, and the values thereof were averaged. Values represent means±S.E.M. for four or five experiments: *$2P < 0.05$ versus hypothyroid, by Student's t test.

	Cytosolic free [Ca^{2+}] (nM)			
Rats	Maximum	Steady state (a)	Isoprenaline (b)	Change (%) [$100 \times (b-a)/a$]
Hypothyroid	338±36	123±16	152±18	+25±3
Hyperthyroid	168±18*	124±12	132±14	+8±4*

hyper-thyroid groups with rising [Ca^{2+}]$_o$. Secondly, the effect of isoprenaline on maximal [Ca^{2+}]$_i$ was relatively highest in the hypothyroid group, although the differences were not significant at 2.5 mM- and 5 mM-Ca^{2+}_o owing to the large scatter of the results. Fig. 4(b) shows that at all [Ca^{2+}]$_o$ values the $RC_{0.5}$ decreased significantly in the hypothyroid group, but no such decrease was seen in the hyperthyroid group.

Effect of high-[K^+]$_o$ depolarization

Partial depolarization by high K^+_o was employed to generate a constant influx of Ca^{2+} through the fraction of slow Ca^{2+} channels which remain open under these circumstances. After re-adjustment of the different Ca^{2+}-transfer systems (slow Ca^{2+} channels, Na^+/Ca^{2+} exchange, Ca^{2+} pumps of sarcolemma and SR) a new steady state at a higher [Ca^{2+}]$_i$ is attained. In Fig. 5 an example is shown of the time course of [Ca^{2+}]$_i$ in a hypothyroid and a hyperthyroid myocyte after addition of 50 mM-K^+_o.

In the hyperthyroid myocyte the [Ca^{2+}]$_i$ gradually rose to 150 nM, which is similar to the results found previously in suspensions [8]. The hypothyroid myocyte behaved completely differently. First there was a large overshoot of [Ca^{2+}]$_i$, which resulted in a clearly visible contracture. Next, [Ca^{2+}]$_i$ decreased to a steady-state value which was not different from that of the hyperthyroid myocyte. When at this stage isoprenaline was added, an increase of [Ca^{2+}]$_i$ was observed in the hypothyroid myocyte, but not in the hyperthyroid myocyte. The results from these experiments are presented in Table 4. It shows a significantly higher maximal response to high [K^+]$_o$ and isoprenaline in the hypothyroid group compared with the hyperthyroid group, but no difference in steady-state [Ca^{2+}]$_i$ between the two groups during high-[K^+]$_o$ exposure.

DISCUSSION

General

One of the major conclusions that can be drawn from this study is that thyroid hormone increases [Ca^{2+}]$_i$ at rest and during contraction in rat cardiomyocytes. Increased Ca^{2+} availability therefore appears to be a major factor in the inotropic effect of thyroid hormone in heart. In addition, these altered [Ca^{2+}]$_i$ values may be involved in the increased metabolic activity induced by thyroid hormone. However, this will be determined by the magnitude of the thyroid-hormone effects on [Ca^{2+}]$_i$ in absolute terms. Reliable determination of these [Ca^{2+}]$_i$ values requires an adequate calibration procedure and minimal side effects of dye loading. Absolute [Ca^{2+}]$_i$ values obtained by using Ca^{2+}-dependent fluorescent dyes have been criticized on several grounds [14–17]. One of these is that [Ca^{2+}]$_i$ may vary with the degree of loading of the dye [14]. We established that, at an intracellular [fura-2] of 40 μM or lower, peak [Ca^{2+}]$_i$ levels during contraction and decay time of the Ca^{2+} transients were not influenced by the dye. We also employed a short loading time (15 min) to minimize accumulation of fura-2 in cell compartments other than the cytosol. Further criticism concerns the pitfalls emerging in calibration procedures with fluorescent dyes, which are related to single-wavelength measurements and calibration *in vitro* [15–17]. By using the ratio method in dual-wavelength measurements and calibration *in vivo*, as we did in this study, these problems are circumvented and quantification becomes more reliable [11]. By applying this calibration method we obtained resting [Ca^{2+}]$_i$ values of 70 nM and peak [Ca^{2+}]$_i$ values of 250 nM during contraction in euthyroid myocytes. The resting [Ca^{2+}]$_i$ obtained in this study with single euthyroid myocytes is in reasonable agreement with that determined in our other study [8] with suspensions (90 nM). Our values of 250 nM during contraction lie in the lower region of previously reported values for cardiomyocytes (150–1000 nM) [18–20]. The wide range of these values may in part be due to differences in the methods of measurement and calibration, but most likely also reflects a true variation in [Ca^{2+}]$_i$ in the different species and types of cell preparation used (cultured or isolated). Indeed, clear differences in force of contraction that can be observed between individual preparations are indicative of such a variation in peak [Ca^{2+}]$_i$.

It should be noted that conclusions concerning the qualitative effects of the thyroid status drawn from the present data, e.g. the hormone-dependent increase in [Ca^{2+}]$_i$ during rest and contraction, do not require knowledge of the absolute [Ca^{2+}]. The ratio of Ca^{2+}-dependent fluorescence at 340 and 380 nm is then used as a dimensionless measure of the [Ca^{2+}]$_i$, and such conclusions therefore remain valid, irrespective of the type of calibration used to get a more quantitative picture.

[Ca^{2+}]$_i$ at rest

We found an increase of 60–70% in resting [Ca^{2+}]$_i$ at a [Ca^{2+}]$_o$ of 1 and 2.5 mM in hyperthyroid monocytes (64 and 80 nM respectively) compared with hypothyroid myocytes (39 and 45 nM respectively). This is in line with results from one of our earlier studies, where hypothyroidism was shown to lead to 30% lower resting [Ca^{2+}]$_i$ in liver cells [21]. No differences in resting [Ca^{2+}]$_i$ levels were found between ferret ventricular muscles of different thyroid status, using aequorin as Ca^{2+} indicator [22]. However, apart from the species difference, there exists some uncertainty about the significance of this result. In the region of low Ca^{2+} concentrations (50–150 nM) which prevail at rest, large errors are easily introduced with the aequorin method used.

The suggestion was made, based on results from our previous studies [21,23], that increases in cytosolic Ca^{2+} could contribute to the increased basal O_2-consumption rate induced by thyroid hormone. Supporting evidence for a positive relationship between [Ca^{2+}]$_i$ and the rate of O_2 uptake in suspensions of cardiac myocytes has recently been presented [17]. Under the assumption that we measured realistic [Ca^{2+}]$_i$ values, the question arises whether significant changes in metabolic rate can be expected in the observed 40–80 nM range. In our earlier study [8] with suspensions of cardiac myocytes, where we found comparable [Ca^{2+}]$_i$ values at rest and during exposure to 50 mM-K^+_o, we could not observe an effect on O_2 consumption when [Ca^{2+}]$_i$ was raised from 90 to 135 nM by 50 mM-K^+_o. We also consider it not

very likely that the activity of Ca^{2+}-dependent ATP-producing or -consuming processes are much stimulated below a $[Ca^{2+}]_i$ of 100 nM. Accordingly, the differences in resting $[Ca^{2+}]_i$ of hypothyroid and hyperthyroid myocytes do not seem sufficiently large enough, in the light of our present knowledge, to induce significant changes in metabolic rate. However, changes in $[Ca^{2+}]_i$ in the 40–80 nM range could affect membrane permeability and conductance [24], for instance the activation of Ca^{2+}-dependent K^+ channels. Whether the increased K^+ efflux, shown by us to occur in hyperthyroid resting muscle [9], is caused by elevated $[Ca^{2+}]_i$ remains, however, to be demonstrated.

The next question that arises concerns the physiological factors that could account for the increase in resting $[Ca^{2+}]_i$ levels in the direction hypothyroid → hyperthyroid. Resting $[Ca^{2+}]_i$ is ultimately determined by the steady state of extrusion by active transport versus leakage (influx) of Ca^{2+} through the plasma membrane. Stimulation of sarcolemmal Ca^{2+}-ATPase in heart by thyroid hormone has been reported [5]. It has also been published that Ca^{2+} influx via slow Ca^{2+} channels and via Na^+/Ca^{2+} exchange is increased by thyroid hormone, but this was measured in beating heart cells [4]. Although in resting fully polarized myocytes the activity of these Ca^{2+}-transfer systems ought to be minimal, some influx may still occur and be thyroid-hormone dependent. It is noteworthy that in the same study [4] addition of the slow Ca^{2+}-channel blocker verapamil, resulting in a complete inhibition of contractility, did not affect the significantly higher Ca^{2+} influx in hyperthyroid cardiac cells. This verapamil-resistant influx component was not defined, but it should be noted that direct stimulation of Ca^{2+} uptake by triiodothyronine through as yet undefined pathways has been reported in liver cells [25] and thymocytes [26].

$[Ca^{2+}]_i$ during electrical stimulation

Electrical stimulation showed a larger rise in $[Ca^{2+}]_i$ in hyperthyroid myocytes than in hypothyroid myocytes. Indications for this of similar but more qualitative nature have come from a recent study of ferret ventricular muscle, using aequorin [22]. Assuming that in contracting mammalian heart cells most of the activating Ca^{2+} cycles between the SR and the contractile proteins [1], two possible explanations can be forwarded for the higher $[Ca^{2+}]_i$ during contractions in hyperthyroid myocytes. Firstly the observation of a higher resting $[Ca^{2+}]_i$ in hyperthyroid cardiac cells would warrant the prediction of a higher Ca^{2+}-filling level of the SR. The positive correlation existing between physiological levels of $[Ca^{2+}]_i$ outside the SR and Ca^{2+} stored in the SR in the presence of ATP has been described by us for skeletal-muscle SR [27]. However, the resting $[Ca^{2+}]_i$ in hyperthyroid myocytes does not increase significantly on raising $[Ca^{2+}]_o$ from 1 to 5 mM, in contrast with the amplitude of the Ca^{2+} transient during contraction, which increases by 60%. This observation argues against a significant role of $[Ca^{2+}]_i$ in the regulation of the capacity for Ca^{2+} release from the SR in this study. A second, more likely, explanation is based on the finding that thyroid hormone stimulates the proliferation of SR in heart [2,3] and skeletal muscle [28,29], thereby increasing the capacity for Ca^{2+} storage and release. In line with this is the observation that in both the hypothyroid and hyperthyroid groups the amplitude of the Ca^{2+} transient increases similarly (60%) on raising $[Ca^{2+}]_o$ from 1 to 5 mM. If differences in the amount of SR underlie the significantly higher Ca^{2+} transients in the hyperthyroid than in the hypothyroid group, one would indeed expect that, by raising $[Ca^{2+}]_o$, the increased Ca^{2+} supply from outside the cell would lead to the same fractional increase in the amount of Ca^{2+} stored in the SR, and the amplitude of the Ca^{2+} transient. The 2–3-fold faster rate of decay of the Ca^{2+} transients once more underscores the increased Ca^{2+}-uptake activity of the SR in the hyperthyroid condition.

Effect of the β-agonist isoprenaline

The major result of experiments with isoprenaline administration was the strikingly diminished effect on both maximal $[Ca^{2+}]_i$ levels during contraction and the decay rate of the Ca^{2+} signal in hyperthyroid myocytes, whereas a clear rise in maximal $[Ca^{2+}]_i$ and a faster decay rate of the Ca^{2+} signal was observed in the hypo- and eu-thyroid groups. The latter is in agreement with previous reports from studies in normal rats [13,30,31]. The extra rise in $[Ca^{2+}]_i$ is ascribed to increased Ca^{2+} release from the SR owing to higher Ca^{2+} filling, and to a lesser extent to decreased Ca^{2+} binding to troponin [28]. Increased Ca^{2+} filling of the SR is effected by β-agonists through increased opening of slow Ca^{2+} channels in the sarcolemma by phosphorylation of the channel and also by increased Ca^{2+} transport into the SR through phosphorylation of phospholamban, which stimulates the Ca^{2+}-ATPase. The stimulation by isoprenaline of these two Ca^{2+}-transfer systems increases the Ca^{2+} flow towards the SR. However, raising cytosolic Ca^{2+} favours inactivation of slow Ca^{2+} channels [32], and it cannot be excluded that the higher Ca^{2+} concentrations during rest and activity in hyperthyroid myocytes result in relatively more inactivation of the slow Ca^{2+} channels, thereby curtailing Ca^{2+}-influx stimulation by isoprenaline. The observation that in both the hypo- and hyperthyroid myocytes the effect of isoprenaline on the amplitude of the Ca^{2+} transients is decreased on raising $[Ca^{2+}]_o$ from 1 to 5 mM, which elevates the Ca^{2+} transients in both groups, supports this idea. With regard to Ca^{2+} transport into the SR during the decay phase of the Ca^{2+} transient, we have shown that in heart SR from hyperthyroid rats the phospholamban/Ca^{2+}-ATPase ratio is much lower (0.31) than in SR from hypothyroid rats (1.69), which explains the low or even absent stimulation by isoprenaline of the $RC_{0.5}$ of hyperthyroid myocytes. Taken together, the evidence suggests that in hyperthyroid myocytes diminished stimulation by isoprenaline of Ca^{2+} flux towards the SR through slow Ca^{2+} channels and SR Ca^{2+}-ATPase could lead to less extra Ca^{2+} filling of the SR and thereby to less effect on the magnitude of the Ca^{2+} transient.

Effect of high-$[K^+]_o$ depolarization

In contrast with the situation during electrical stimulation, where the magnitude of the Ca^{2+} transient is largely determined by the amount of Ca^{2+} stored in the SR, the $[Ca^{2+}]_i$ during high-$[K^+]_o$ depolarization is ultimately determined by the Ca^{2+}-influx/efflux balance across the sarcolemma. In high-$[K^+]_o$ depolarization $[Ca^{2+}]_i$ will rise through opening of a proportion of slow Ca^{2+} channels. The equal $[Ca^{2+}]_i$ levels in hypothyroid and hyperthyroid myocytes during prolonged high-$[K^+]_o$ exposure indicate similar influx/efflux balance of Ca^{2+} across the outer membrane. The large overshoot of $[Ca^{2+}]_i$ in hypothyroid myocytes during the first 5 min of high-$[K^+]_o$ exposure is at present not understood. It could indicate that inactivation of slow Ca^{2+} channels takes place more slowly in the hypothyroid group, or alternatively that Ca^{2+} uptake into the SR is insufficient to buffer the initially enhanced Ca^{2+} influx. Again, a significantly higher $[Ca^{2+}]_i$ rise was found after isoprenaline treatment in $[K^+]_o$-depolarized hypothyroid myocytes, despite an equal initial $[Ca^{2+}]_i$ steady state in hypo- and hyper-thyroid myocytes. This eliminates the possibility in this case that higher $[Ca^{2+}]_i$ levels are responsible for a larger proportion of isoprenaline-resistant Ca^{2+} channels in the hyperthyroid group, as was proposed for the situation during contraction. It indicates that factors still unknown controlled by thyroid hormones influence slow-Ca^{2+}-

channel behaviour and thereby the Ca^{2+}-influx/efflux balance across the outer membrane.

Although clearly needing further study, the general picture emerging from the present results shows that interventions which alter the Ca^{2+}-influx/efflux balance ($[Ca^{2+}]_o$ elevation, high $[K^+]_o$, β-agonists) have less impact on Ca^{2+} homoeostasis when thyroid-hormone activity increases.

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