On the mechanism of the reduction by thyroid hormone of β -adrenergic relaxation rate stimulation in rat heart

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The effects of β -adrenergic stimulation on the relaxation rate and the Ca^{2+} -transport rate in sarcoplasmic reticulum of hypothyroid, euthyroid and hyperthyroid rat hearts were studied. Administration of isoproterenol ($0.1 \mu\text{M}$) to perfused, electrically stimulated hearts (5 Hz) caused a decrease in the half-time of relaxation ($\text{RT}_{1/2}$) the extent of which depended on the thyroid status, i.e. hypothyroid (-24%), euthyroid (-19%) or hyperthyroid (-8%). A similar decreasing effect was found for the stimulation of Ca^{2+} transport in isolated SR by cyclic AMP and protein kinase, i.e. hypothyroid (75%), euthyroid (37%) and hyperthyroid (20%). These alterations were not due to differences in endogenous protein kinase activity or cyclic AMP production. Estimations of Ca^{2+} -ATPase and phospholamban (PL) content of the sarcoplasmic reticulum were obtained by measurement of the phosphorylated forms of Ca^{2+} -ATPase (E-P) and phospholamban (PL-P) followed by electrophoresis and autoradiography. A 3-fold decrease of PL-P, accompanied by a 2-fold increase of E-P per mg of protein was observed in sarcoplasmic reticulum preparations in the direction hypothyroid $\rightarrow$ hyperthyroid. Consequently the E-P/PL-P ratio increased from 0.32 (hypothyroid), through 0.81 (euthyroid) to 1.69 (hyperthyroid). In spite of certain limitations inherent to quantification of Ca^{2+} -ATPase and phospholamban by their phosphorylated products, these data provide strong evidence that during thyroid-hormone mediated cardiac hypertrophy, with concomitant proliferation of the sarcoplasmic reticulum, the relative amount of phospholamban decreases with respect to Ca^{2+} -ATPase. This could provide an explanation for the observed gradual diminishment of the β -adrenergic effect on the relaxation rate when cardiac tissue is exposed to increasing amounts of thyroid hormone.

INTRODUCTION

Thyroid hormone and catecholamines are major regulators of myocardial contractility. This regulation is achieved at least partly by increasing the capacity for Ca^{2+} transport into the sarcoplasmic reticulum (SR) resulting in acceleration of the relaxation rate. The mechanisms by which these hormones act on SR, however, are different. Thyroid hormone is supposed to accelerate relaxation rate in heart by increasing the number of SR Ca^{2+} pumps, which process takes one or more days [1–3]. Catecholamines, mainly by their β -agonistic action, stimulate relaxation rate within a few seconds by elevating intracellular cyclic AMP. In the presence of cyclic AMP and cyclic AMP-dependent protein kinase (PK) a protein of approx. M_r 22 000 from the SR, phospholamban (PL), has been shown to become specifically phosphorylated [4,5]. Interaction between the SR Ca^{2+} pump and phosphorylated phospholamban is assumed to result in a higher Ca^{2+} -ATPase activity and Ca^{2+} -uptake rate.

We found in an earlier study that the effects of the β -receptor agonist isoproterenol (IP) on Ca^{2+} transients of electrically-stimulated cardiac myocytes are inversely related to the documented changes in β -receptor density in heart tissue occurring with alterations in the thyroid status [6]. In the hypothyroid group a large accelerating effect was observed on the Ca^{2+} rise and decay in the

presence of IP and hardly any effect on these parameters was seen in the hyperthyroid group. These observations agree well with studies on IP-stimulated relaxation rate in slow skeletal muscle [7] and papillary muscle [8]. This led to the suggestion that the relaxant effect of IP does not occur in hyperthyroidism because the cellular mechanisms governing relaxation are already saturated before exposure to IP [8]. However, until now, these cellular mechanisms were not identified. According to the available literature there is no evidence for lower cyclic AMP levels [9,10] or PK activity [11] during β -receptor stimulation in hyperthyroid heart. Therefore, a prime candidate that could be responsible for the different responses to IP in altered thyroid states is the SR. The few studies which considered cyclic AMP-mediated Ca^{2+} uptake in SR from heart muscle of different thyroid states are not unanimous. Unchanged as well as abolished Ca^{2+} -uptake stimulation by cyclic AMP and PK in SR from hyperthyroid heart has been reported [11,12]. Therefore, we investigated this subject in more detail by studying β -agonistic effects on whole heart (relaxation rate) and on SR (Ca^{2+} uptake) and by determining the relative amounts of Ca^{2+} -ATPase and PL in different thyroid states. We present evidence that the progressive reduction of β -agonistic stimulation of the relaxation rate of beating rat hearts in the direction hypothyroid $\rightarrow$ hyperthyroid correlates well with a similar decrease of PL with respect to Ca^{2+} -ATPase in heart SR.

Abbreviations used: SR, sarcoplasmic reticulum; $\text{RT}_{1/2}$ half-time of relaxation; PK, cyclic-AMP dependent protein kinase; PL(-P), (phosphorylated) phospholamban; E-P, phosphorylated intermediate of Ca^{2+} -ATPase; IP, isoproterenol; T_3 , L-tri-iodothyronine.

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Part of this work has been presented at the XVIIIth European Conference on Muscle and Motility at Abano Terme (Padua).

MATERIALS AND METHODS

Animals

The experiments were carried out using fed male Wistar rats in the weight range 250–280 g. They were fed a normal laboratory chow (Hope Farms, Linschoten, The Netherlands). To induce hypothyroidism, animals were maintained on a low-iodine diet (Hope Farms, Linschoten, The Netherlands) with 2% (w/v) KClO_4 in their drinking water for a period of 6 weeks [13]. Hyperthyroidism was induced by daily subcutaneous injections of L-tri-iodothyronine (T_3) (20 μg T_3 /100 g body weight) for 1 week [13].

Heart perfusion

Hearts were perfused by the Langendorff technique [14] with oxygenated Tyrode solution at a constant flow rate of 10 ml/min at 37 °C. The solution contained (mM): NaCl (128), KCl (4.7), CaCl_2 (1.4), MgCl_2 (1.0), NaHCO_3 (20), NaH_2PO_4 (0.4) and glucose (11.1). Hearts were continuously paced at 5 Hz by 10 ms electrical impulses at suprathreshold voltages. Heart force was measured isovolumetrically. This was achieved by introducing a latex balloon attached to a stainless steel needle into the left ventricular chamber. Left ventricular pressure development was then measured at optimal filling pressure (1.6–2.6 kPa) through the use of a Millar PC-380 pressure transducer (Millar Instruments Inc.) connected to the stainless steel needle. The pressure signal was registered by a chart recorder (Gould 2200). IP was administered by continuous infusion (0.05 ml/min) via a side arm which entered the main perfusion stream just before the attachment of the aorta to the cannula.

Calcium transport

A microsomal fraction from heart enriched in SR was obtained essentially according to the method described by Harigaya & Schwartz [15]. Ca^{2+} -uptake rates were determined in freshly prepared SR by a modification of the previously described Ca^{2+} -stat method [16]. Instead of the Ca^{2+} -selective electrode, this improved method uses the fluorescent Ca^{2+} -indicator fura-2 [17] to monitor the free $[\text{Ca}^{2+}]$ in the uptake medium in the micromolar range with greater sensitivity and a more rapid response time. The Ca^{2+} signal from the fluorimeter is coupled to an automatic titrator and autoburette (Radiometer, Copenhagen) that effectively clamps the preset $[\text{Ca}^{2+}]$ in the medium during active Ca^{2+} -uptake by infusion of a concentrated Ca^{2+} solution (0.5 mM). Recording of the infused Ca^{2+} gives a Ca^{2+} -uptake curve at constant external $[\text{Ca}^{2+}]$. The uptake rate is calculated from the slope of the curve, which is essentially linear during the first minute. Ca^{2+} -uptake activity was maximal at Ca^{2+} concentrations higher than 3 μM . The assay was carried out in a quartz cuvette in 2 ml of histidine buffer (40 mM) containing 5 mM-sodium oxalate, 100 mM-KCl, 1 mM- MgCl_2 , 10 mM- NaN_3 , 20 μM -EGTA and 0.5 μM -fura-2, pH 7.0, using an Aminco-Bowman fluorimeter equipped with a thermostatted cuvette holder (25 °C). Excitation and emission wavelengths were 340 and 490 nm, respectively. SR (0.1–0.4 mg) was preincubated

for 5 min in the assay buffer, without oxalate, with and without PK (400 μg) or cyclic AMP (1 μM) and 10 mM-ATP. The reaction was started by adding sodium oxalate (5 mM) and CaCl_2 (2.5–15 μM), resulting in Ca^{2+} concentrations ranging from 1–5 μM . The Ca^{2+} concentrations were calculated from the fluorescence signal, using the equation $[\text{Ca}^{2+}] = K_d (F - F_{\min.}) / (F_{\max.} - F)$. Determination of the K_d of fura-2 for Ca^{2+} at 25 °C yielded a value of 350 nM, following the procedure described by Grynkiewicz *et al.* [17].

Determination of phosphorylated intermediate of SR Ca^{2+} -ATPase

Cardiac SR (0.375 mg/ml) was incubated in 1 mM- MgCl_2 /120 mM-KCl/40 mM-histidine/HCl buffer (pH 7.0)/125 μM - CaCl_2 or 1 mM-EGTA in a total volume of 0.2 ml at 4 °C. The reaction was started by the addition of 10 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.2 mCi/ μmol) and terminated after 30 s with 2 ml ice-cold 10% trichloroacetic acid containing 1 mM-ATP and 25 mM- KH_2PO_4 . The pellet after centrifugation (1000 g, 10 min) was washed twice more with the trichloroacetic acid mixture. The final pellet was taken up in 150 μl of solubilization buffer (see below). A 10 μl aliquot was assayed for radioactivity by liquid scintillation counting, while the remaining volume was used for gel electrophoresis. Boiling of the phosphorylated samples, or exposure to hydroxylamine (1 mM, 30 °C) abolished all phosphate incorporation.

Determination of cyclic AMP-dependent SR phosphorylation

Cardiac SR was phosphorylated at 25 °C for 2 min in a total volume of 150 μl according to the following procedure. Cardiac SR (0.3 mg/ml) was incubated in 40 mM-histidine/HCl buffer (pH 7.0) with 0.4 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (0.125 mCi/ μmol), 2 mM- MgCl_2 , 120 mM-KCl and 25 mM-NaF, with or without 0.2 mg/ml of cyclic AMP-dependent PK and 1 μM -cyclic AMP. Autophosphorylation of cyclic AMP-dependent PK was determined under the same conditions except that the SR was omitted. The reaction was terminated with 2 ml of ice-cold 10% trichloroacetic acid containing 1 mM-ATP and 25 mM- KH_2PO_4 . The pellet after centrifugation (1000 g, 10 min) was washed twice more with the trichloroacetic acid mixture. The final pellet was taken up in 120 μl of solubilization buffer. A 10 μl aliquot was assayed for radioactivity by liquid scintillation counting, while the remaining part was used for gel electrophoresis. The cyclic AMP-dependent phosphate incorporation was more than 90% resistant to boiling or exposure to hydroxylamine (1 mM, 30 °C).

SDS/polyacrylamide-gel electrophoresis

The analysis of cyclic AMP-dependent phosphorylated products by SDS/polyacrylamide-gel electrophoresis was performed using the buffer system of Laemmli [18] and a gradient gel of 7.5–15%, with a 5% stacking gel. Samples for electrophoresis were suspended in solubilization buffer consisting of 2% (w/v) SDS, 30 mM-Tris/HCl (pH 7.0), 20 mM-dithiothreitol, 10% glycerol and 0.00025% (w/v) Bromophenol Blue. The pellet was incubated at 20 °C for 5 min. An aliquot of 40 μg of protein was applied to the gel. After electrophoresis, the proteins were made visible by staining the gels with Coomassie Brilliant Blue.

The analysis of phosphorylated intermediate of Ca^{2+} -

ATPase by polyacrylamide-gel electrophoresis was performed essentially as described by Lichtner & Wolf [19] using a 6% gel and the following buffer system (pH 2.4): 93.8 mM-citric acid, 12.4 mM-phosphoric acid, 12 mM-Tris buffer, 0.1% (w/v) lithium dodecyl sulphate. Samples for electrophoresis were suspended in the sample buffer (pH 2.4) consisting of lithium dodecyl sulphate (2%, w/v), citric acid (50 mM), phosphoric acid (6.5 mM), Tris/HCl (6.3 mM), EDTA (1 mM) and dithiothreitol (10 mM). Solubilization was achieved after incubation for 20 min at 5 °C. Samples were diluted (1:1) in a solution with sucrose (2 mM) and Pyronin Y (40 μ g/ml). An aliquot of 50 μ g of protein was applied to the gel.

The M_r values of proteins were determined by comparing their relative mobilities to those of the following protein standards: myosin (200 000), β -galactosidase (116 250), phosphorylase *b* (94 000), bovine serum albumin (66 200), ovalbumin (43 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500) and lysozyme (14 400).

Autoradiography

After electrophoresis of the cyclic AMP-dependent phosphorylation products, the slab gels were fixed, stained with Coomassie Brilliant Blue and dried. The dried gels were placed in Kodak-X-omatic cassettes with intensifying screen and Fuji X-ray films layered over the gels. Exposures were for 5 days at -80 °C.

The gels containing the phosphorylated intermediate of Ca^{2+} -ATPase were subjected to autoradiography directly following electrophoresis, after sealing the wet gels with cellophane. Exposures were for 2 days at -80 °C. For the identification of the proteins after autoradiography, the gels were fixed, stained and dried.

Other determinations

Protein concentrations were determined according to ref. [20] with bovine serum albumin (Sigma Chemical Co.) as standard.

Materials

ATP (disodium salt), cyclic AMP (sodium salt), bovine heart cyclic AMP-dependent protein kinase, bovine heart phosphodiesterase 3'-5'-cyclic nucleotide, bovine heart protein kinase inhibitor, L-isoproterenol-*d*-bitartrate, dithiothreitol and L-tri-iodothyronine were all purchased from Sigma, St. Louis, MO, U.S.A.; [γ - ^{32}P]-ATP (3 mCi/ μ mol) was from Amersham Nederland BV, Houten, The Netherlands; fura-2 was from Molecular Probes, Eugene, OR, U.S.A.; acrylamide, *NN'*-methylenebisacrylamide and protein molecular weight standards were from Bio-Rad Laboratory BV, Utrecht, The Netherlands. Sodium and lithium dodecyl sulphate were from BDH Chemicals, Poole, Dorset, U.K., and Coomassie Brilliant Blue and Pyronin Y were from Serva, Heidelberg, Germany. All other chemicals were of the highest purity grade available.

Statistical analysis

Statistical significance of differences between means was determined using a two-tailed Student's *t*-test and designated at $2P < 0.05$.

RESULTS

Mechanical measurements

Contractile effects of IP (0.1 μM) are shown in Fig. 1(a). Initially there was a large inotropic response with a rise in force of nearly 400%. Thereafter, force gradually decreased and reached a steady state after 25 s at a level 50% higher than resting levels. The $\text{RT}_{1/2}$ was measured during this period. Fig. 1(b) shows typical recordings from hypothyroid and hyperthyroid preparations before and during IP infusion. Euthyroid preparations produced an intermediate picture (not shown). The collected data from hypothyroid, euthyroid and hyperthyroid hearts are presented in Table 1. It shows that the $\text{RT}_{1/2}$ considerably decreased in the direction hypothyroid $\rightarrow$ hyperthyroid. IP infusion caused significant decreases in $\text{RT}_{1/2}$ in all three groups. However, the magnitude of the IP effect was inversely related to that of thyroid hormone.

SR measurements

Since Ca^{2+} uptake into the SR is a major factor in determining relaxation rate, we measured this parameter in heart SR isolated from hypothyroid, euthyroid and hyperthyroid rats. Ca^{2+} uptake by SR, pretreated with and without cyclic AMP plus PK, was measured at different Ca^{2+} concentrations as determined by fura-2 fluorescence. Significant stimulation of Ca^{2+} uptake by cyclic AMP/PK was seen at a Ca^{2+} concentration of 1 μM . Fig. 2 shows a decrease in the percentage of Ca^{2+} -uptake stimulation at all Ca^{2+} levels in the direction hypothyroid $\rightarrow$ hyperthyroid. The results agree well with those on mechanical function. The stimulation of Ca^{2+} uptake by cyclic AMP/PK in absolute terms, at saturating Ca^{2+} concentrations, is shown in Table 2. Whereas the difference between hypothyroids and hyperthyroids in untreated controls is more than 100%, greater stimulation of Ca^{2+} uptake by cyclic AMP/PK reduced this difference to 60%. The occurrence of

Fig. 1. Effect of IP on heart contractility

(a) Force recording from isovolumetrically contracting rat hearts (5 Hz) during perfusion; (b) single contractions of hypothyroid and hyperthyroid rat hearts in the absence (1) and presence (2) of 0.1 μM -IP.

Table 1. Effect of IP on relaxation time of contracting rat hearts

Half relaxation time ($RT_{1/2}$) measurements of perfused isovolumetrically contracting rat hearts (5 Hz) are given for different thyroid states in the presence and absence of IP. The values presented are the means $\pm$ S.E.M. of (*n*) determinations. * $2P < 0.05$, ** $2P < 0.02$ versus euthyroids.

Status	Addition...	$RT_{1/2}$ (ms)		Change in $RT_{1/2}$ (%)
		None	0.1 μ M-IP	
Hypothyroid (5)		45 $\pm$ 1**	35 $\pm$ 1**	-24 $\pm$ 1*
Euthyroid (6)		31 $\pm$ 2	25 $\pm$ 1	-19 $\pm$ 2
Hyperthyroid (9)		26 $\pm$ 1**	24 $\pm$ 1	-8 $\pm$ 1**

Fig. 2. Effect of cyclic AMP and PK on Ca^{2+} transport in cardiac SR

Ca^{2+} -transport rates were measured at different free $[Ca^{2+}]$ using a Ca^{2+} -stat technique with fura-2 as Ca^{2+} monitor. Ca^{2+} uptake was assayed in the absence (controls, open symbols) and presence (closed symbols) of cyclic AMP (1 μ M) and PK (200 μ g/ml). The values are expressed as a percentage of the maximum uptake rate (V_{max}) of the controls (100%) from each group.

endogenous cyclic AMP/PK activity in microsomal preparations from heart has been suggested [12]. Therefore, we investigated whether an increasing endogenous cyclic AMP/PK activity in the direction hypothyroid $\rightarrow$ hyperthyroid could be responsible for, or at least contribute to, the observed differences in Ca^{2+} -uptake stimulation between the groups. In that case, PL should be nearly maximally phosphorylated in the hyperthyroid group and less so in the euthyroid and especially the hypothyroid groups.

Addition of cyclic AMP alone (1 μ M) or phosphodiesterase (200 μ g/ml) did not influence Ca^{2+} uptake in any of the experimental groups. We found furthermore that compared to untreated controls (100%), the addition of PK inhibitor (500 μ g/ml) resulted in only a negligible effect on Ca^{2+} uptake in the euthyroid ($-6 \pm 3\%$) ($n = 4$) and the hyperthyroid groups ($-2 \pm 2\%$) ($n = 2$). On the other hand, Ca^{2+} -uptake stimulation by cyclic

AMP (1 μ M) plus PK (50 μ g/ml) in the euthyroid group ($+47 \pm 9\%$) ($n = 3$) was effectively blocked by PK inhibitor ($99 \pm 6\%$) ($n = 2$). All values represent means $\pm$ S.E.M. These results do not point to significant intrinsic cyclic AMP/PK activity in our SR preparations.

Phosphoproteins in cardiac SR

Thyroid hormone causes hypertrophy of cardiac cells including enlargement of the SR with a concomitant increase of SR proteins such as Ca^{2+} ATPase and PL [1,6]. The experiments described in this section were devised to see whether the relative proportions of Ca^{2+} -ATPase and PL change depending on the thyroid status. To obtain an estimate of the amounts of Ca^{2+} -ATPase and P-L, the proteins were maximally phosphorylated - E-P and PL-P respectively - under appropriate conditions and subjected to electrophoresis. Fig. 3(a) shows an example of the protein profile on a 7.5-15%

Table 2. Effect of cyclic AMP/PK on maximum Ca^{2+} uptake rate in SR of rat heart

The experimental details were as described in the legend to Fig. 2. Ca^{2+} -uptake rate was measured at saturating Ca^{2+} concentrations ($3 \mu\text{M}$ - Ca^{2+} or higher). The values represent means $\pm$ S.E.M. for (*n*) paired controls and cyclic AMP/PK-treated SR samples. **P* < 0.05, ***P* < 0.02 versus euthyroids; †*P* < 0.02 versus hypothyroids.

Status	Addition ...	Ca ²⁺ -uptake rate (nmol/min per mg of protein)		Change in Ca ²⁺ - uptake rate (%)
		None (controls)	cAMP (1 μM) + PK (200 $\mu\text{g/ml}$)	
Hypothyroid (4)		6.3 $\pm$ 0.4	10.8 $\pm$ 0.5	75 $\pm$ 11*
Euthyroid (4)		8.9 $\pm$ 1.1	12.0 $\pm$ 1.3	37 $\pm$ 8
Hyperthyroid (5)		14.4 $\pm$ 0.7**	16.6 $\pm$ 1.6†	20 $\pm$ 3*

Fig. 3. SDS/polyacrylamide-gel electrophoresis and autoradiography of SR isolated from rat heart

Photographs of (a) Coomassie Brilliant Blue-stained gel and (b) autoradiogram of SR isolated from hypothyroid, euthyroid and hyperthyroid hearts are shown. Phosphorylations of PL plus (+) and minus (−) cyclic AMP and PK are indicated. The concentration of SDS was 2%; and 5–15% gels were used.

gradient SDS/polyacrylamide gel of equal protein amounts of cardiac SR from hypothyroid, euthyroid and hyperthyroid rat hearts. The pattern of protein bands of euthyroid and hyperthyroid samples was similar. Hypothyroid samples revealed a protein with an M_r of 94000, which was virtually absent in the other two groups. By scanning the gels it could be shown that contamination by this protein in the hypothyroid group varied between 5 and 11%. This would slightly underestimate the PL-P and E-P quantities by the same percentages in this group, but hardly influence the E-P/PL-P ratio (see below). The cyclic AMP/PK-treated samples showed an additional protein band with an M_r of 66000, which could represent the added PK.

Fig. 3(b) presents an illustrative autoradiogram of cardiac SR from all three groups, phosphorylated in the presence and absence of cyclic AMP/PK. All three groups showed cyclic AMP/PK-dependent phosphorylation of proteins with M_s of approx. 23000 and 12000. According to published values [21–25] the 23000- M_r spot corresponds with that of the dimeric protein PL,

whereas the M_r -12000 spot corresponds with that of a monomeric dissociation product of PL which is produced during solubilization. This was confirmed by boiling the SR in solubilization buffer for 5 min which dissociates the M_r -23000 protein completely, moving all the radioactivity to the M_r -12000 spot (not shown). The intensity of both the M_r -23000 and M_r -12000 spots strongly decreased in the direction hypothyroid $\rightarrow$ hyperthyroid. Scanning of the autoradiogram in Fig. 3(b) revealed a decrease in intensity of 65%, in the direction hypothyroid $\rightarrow$ hyperthyroid, for both the M_r -23000 and M_r -12000 proteins. Basal phosphorylation (Fig. 3b, minus (−) panels) of the M_r -23000 and M_r -12000 protein was 1% or less in all groups. Only in the hypothyroid sample was a prominent phosphorylation spot consistently seen at a position corresponding with the M_r -94000 band which was revealed by Coomassie staining of the gel (see above). Most probably it represents phosphorylase which is phosphorylated by phosphorylase kinase in the presence of cyclic AMP. We speculate that the hypothyroid preparation was somewhat contami-

nated with glycogen particles containing phosphorylase kinase. Glycogen particles are known to be associated with the SR [26].

The intensity of the M_r -66000 spot was also greater in the presence of cyclic AMP/PK (see Fig. 3a) than in its absence. This spot corresponds with the putative relative mobility of PK (see Coomassie stain) and the intensity increase is likely explained by autophosphorylation. Very interestingly, there is a M_r -20000 spot in all three groups, which is cyclic AMP-dependent, the intensity of which in this case increases in the direction hypothyroid $\rightarrow$ hyperthyroid. High cyclic AMP/PK-independent phosphorylation is seen at the M_r -43000 spot

Fig. 4. Autoradiogram of a lithium dodecyl sulphate/polyacrylamide gel of SR isolated from rat heart

A photograph of an autoradiogram of electrophoresed SR from hypothyroid, euthyroid and hyperthyroid rat heart is shown. Samples used were the same as those in Fig. 3. Phosphorylation of Ca^{2+} -ATPase in the presence of Ca^{2+} (125 μM) (+) and in the absence of Ca^{2+} (-) plus EGTA (1 mM) is indicated. Protein profiles of these gels and those shown in Fig. 3(a) were identical. The lithium dodecyl sulphate concentration was 2%, and a 6% gel was used.

which increases in the direction hypothyroid $\rightarrow$ hyperthyroid.

Quantification of the phosphorylated intermediate of Ca^{2+} -ATPase (E-P) necessitated adaptation of the buffer system and pH (to a value of 2.4), as E-P is labile at neutral pH. This modification resulted in at least 80% recovery on the gel of the radioactive counts present in the trichloroacetic acid precipitate from the phosphorylation assay. Fig. 4 shows that when the assay was carried out in the presence of Ca^{2+} , virtually all radioactivity was localized at approx. M_r -100000 in all groups, which suggests exclusive phosphorylation of the Ca^{2+} -ATPase under these conditions. This was further confirmed by analysis of purified SR Ca^{2+} -ATPase from rat skeletal muscle which comigrated with the phosphorylated compound of heart SR (not shown). The Ca^{2+} dependency of this phosphorylation was shown by the absence of radioactivity at the M_r -100000 spot when the assay was carried out in the presence of EGTA (1 mM). The samples used in Figs. 3 and 4 are the same, and in contrast with the PL results of Fig. 3(b), the intensity of the radioactive spots of E-P increased here (Fig. 4) in the opposite way, i.e. in the direction hypothyroid $\rightarrow$ hyperthyroid, by a factor of 2. Scanning the autoradiograms of the samples shown in Figs. 3(b) and 4 indicated that the E-P/PL-P ratio changed from 0.5 (hypothyroid) to 2.8 (hyperthyroid). The results of the phosphorylation assays for the different groups are summarized in Table 3. The quantity of E-P appeared to be more than doubled in hyperthyroid SR compared with the hypothyroid preparations. Regarding the amounts of PL-P, the opposite was observed: PL-P was decreased by more than 50% in hyperthyroid SR compared to hypothyroid SR. It can also be seen that in the euthyroid preparation the amounts of PL-P and E-P are reasonably in balance with each other. By taking the E-P/PL-P ratio, which eliminates the potential error of differences in SR purity in the three experimental groups, it can be convincingly shown that the amount of PL-P with respect to E-P strikingly decreases in the direction hypothyroid $\rightarrow$ hyperthyroid.

DISCUSSION

The results of this study provide a mechanism that may explain the difference in contractile responsiveness to maximal stimulation by the β -receptor agonist IP between hyperthyroid and hypothyroid rat heart. The study shows that the relaxant effect of IP is increased in hypothyroid heart and decreased in hyperthyroid heart

Table 3. Phosphorylation of Ca^{2+} -ATPase and PL in SR isolated from rat heart

Ca^{2+} -ATPase and PL contents were estimated by measurement of the phosphorylated products as described in the Materials and methods section. E-P, phosphorylated intermediate of Ca^{2+} -ATPase; PL-P, phosphorylated phospholamban. Values represent means $\pm$ S.E.M. of (*n*) determinations. * $2P < 0.05$; ** $2P < 0.02$ versus euthyroids.

Thyroid status	Protein phosphorylation (nmol/mg of protein)		E-P/PL-P
	E-P	PL-P	
Hypothyroid (7)	$0.099 \pm 0.005^{**}$	0.347 ± 0.037	$0.32 \pm 0.05^{**}$
Euthyroid (8)	0.208 ± 0.025	0.296 ± 0.046	0.81 ± 0.11
Hyperthyroid (7)	0.248 ± 0.038	$0.151 \pm 0.020^{*}$	$1.69 \pm 0.20^{**}$

relative to the euthyroid (control) situation. These observations confirm and extend those of Wei *et al.* [8] and Guarnieri *et al.* [11] in papillary muscles of euthyroid and hyperthyroid rats. Until now, no satisfactory explanation has been given for this phenomenon. Similar results of our earlier study [6], obtained by measuring changes in cytosolic free Ca^{2+} with the fluorescent indicator fura-2 in electrically stimulated cardiac myocytes, already indicated the involvement of intrinsic changes in Ca^{2+} transport. In that study, we pointed out that the changes in response to IP in different thyroid states were not likely explained by differences in β -receptor number, cyclic AMP levels or PK activity. β -receptor numbers are even inversely related to the relaxant effect of IP in different thyroid states [27]. Accordingly, all the available evidence pointed to the SR as origin of the thyroid-hormone-dependent effect of IP on relaxation rate. Our results indeed show that the increase of Ca^{2+} uptake in isolated SR by cyclic AMP/PK is higher in the hypothyroid group and lower in the hyperthyroid group with respect to the euthyroid controls. Absence of Ca^{2+} -uptake stimulation in SR by cyclic AMP/PK in a microsomal fraction of hyperthyroid rat heart was found by Guarnieri *et al.* [11]. Limas [12], however, found a higher activity in SR from hyperthyroids when adding cyclic AMP alone and an unchanged effect compared to euthyroid controls when adding cyclic AMP plus PK. His conclusion was that a higher endogenous protein kinase activity in the hyperthyroid microsomal fraction was responsible for the larger stimulation in the hyperthyroid preparation. We found no evidence for endogenous PK activity, as addition of PK inhibitor had no effect on Ca^{2+} uptake in SR from euthyroid and hyperthyroid rats, while it was shown that Ca^{2+} -uptake stimulation by added cyclic AMP/PK was completely inhibited by PK inhibitor in euthyroid SR preparations. It is commonly accepted that the β -agonistic stimulation of Ca^{2+} transport in SR is mediated by phosphorylation of the M_r -23000 protein PL. It is also known that thyroid hormone induces hypertrophy of the cardiac cell, with concomitant proliferation of the SR. The results of Table 2 indicate that this is accompanied by a higher specific Ca^{2+} -uptake activity of the SR, which has also been found by others [1–3]. In addition, it was shown very recently that thyroid hormone markedly increases the level of mRNA coding for SR Ca^{2+} -ATPase in the rat heart [28].

The results of Table 3 show that the amounts of E-P and PL-P change in opposite directions on the trajectory hypothyroid $\rightarrow$ hyperthyroid. While E-P is continuously increasing, PL-P decreases, with a corresponding increase in the E-P/PL-P ratio of 0.3 (hypothyroid) to 1.7 (hyperthyroid).

The question could be raised as to whether the E-P and PL-P content as determined in this study represent the quantities of Ca^{2+} -ATPase and PL in the SR preparations. This presupposes that both molecules were maximally phosphorylated and also that this phosphorylation is stoichiometric. Regarding the first point, it cannot be excluded that our isolated preparations were already partially phosphorylated. However, Ca^{2+} -ATPase is not phosphorylated in the absence of ATP. In the case of PL, it has been shown that endogenous basal phosphorylation is at most 15% of the maximally-stimulated value, i.e. in the presence of IP [5]. This result was obtained with endogenous phosphate activity

inhibited by NaF [29] and endogenous phosphorylation in our preparations will therefore be less or even absent, as we isolated in the absence of NaF. So it seems unlikely that partial phosphorylation has significantly influenced our results. Regarding the stoichiometric character of the phosphorylation, it has been shown that cyclic AMP/PK-stimulated phosphorylation occurs virtually exclusively on a serine residue of PL which is distinct from the phosphorylation catalysed by calmodulin/PK. Furthermore, phosphorylation catalysed by the two kinases occurs independently and additively [30]. Taken together, the available evidence indicates that the measurement of PL and Ca^{2+} -ATPase by their phosphorylated products provides a reliable measure for the quantities of these proteins. Assuming this, the results imply that during thyroid hormone-induced proliferation of the SR the net synthesis of PL progressively lags behind that of Ca^{2+} -ATPase resulting in a change in the relative proportions of the Ca^{2+} -ATPase and PL. In the ideal situation each Ca^{2+} -ATPase molecule interacts with a PL molecule enabling stimulation by cyclic AMP/PK. Estimations of the amounts of Ca^{2+} -ATPase and PL in heart SR by measurement of the phosphorylated products suggest a 1:1 stoichiometry [31]. In our study, 0.21 nmol of E-P/mg of protein and 0.3 nmol of PL-P/mg of protein were measured in SR from normal rats. Our data on E-P and PL-P content in microsomal preparations of different thyroid states suggest the possibility that in the direction hypothyroid $\rightarrow$ hyperthyroid, increasingly fewer PL molecules might be available for interaction with Ca^{2+} -ATPase during β -adrenergic stimulation. This would provide a simple explanation for the different β -adrenergic effects in heart tissue on the Ca^{2+} transients [6], the relaxation process and Ca^{2+} -uptake rates in altered thyroid states.

In rat soleus, a predominantly slow skeletal muscle, we observed a decreasing stimulatory effect of IP on the relaxation rate in the transition from hypothyroid to hyperthyroid, which also results in the conversion of slow into fast fibres [7,33,34]. Together with the present data and the recent observations that PL is present in slow but not in fast fibres [35], and that skeletal muscle and cardiac PL are products of the same gene [36], these data are highly suggestive of a down-regulation of PL gene expression by thyroid hormone in slow skeletal muscle as well as in heart, and they deserve further study.

This work was supported by grant 900-540-147 from the Netherlands Organization for Research (NWO). The skilled technical assistance of Mrs M. J. Zuidwijk and Mrs E. A. M. Marinus is gratefully acknowledged.

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Received 30 August 1988/7 November 1988; accepted 6 December 1988