Effect of various PKC modulators on the sulfation of cell layer GAGs of Caco-2 cells. To test the hypothesis that activated PKC may exert a control on GAG sulfation, the effect of PMA, a phorbol ester known to activate the enzyme by promoting its translocation from the cytosol to the membrane[24], was next tested on ^{35}S-sulfate incorporation into cell layer GAGs of confluent Caco-2 cells. The table indicates that a 1-h treatment of Caco-2 cells with 100 ng/ml of PMA, a concentration shown to be maximally effective on PKC activation[24], resulted in a $47 \pm 3\%$ decrease in the sulfation of cell layer GAGs. Moreover, the table shows that 1,2-dioctanoyl-glycerol (100 µg/ml), a permeant diacylglycerol recently shown to induce in MCF-7 human breast cancer cells a discrete but significant translocation of PKC from cytosol to membrane[25], reduced GAG sulfation by $31 \pm 5\%$. By contrast, 4α-phorbol 12, 13 didecanoate (4α-PDD, 100 ng/ml), an inactive phorbol ester, proved ineffective in modifying this process (table); this provides an assessment of the specificity of the effect of PMA. Our finding that PKC activators decreased GAG sulfation whereas an inactive phorbol ester was without effect on this process is consistent with the results reported elsewhere[4, 26]. Such a finding further supports the hypothesis that the enzyme, once activated, may exert a negative control on GAG sulfation.

In conclusion, our results provide evidence that the spontaneous differentiation of Caco-2 cells is associated with an increase in the sulfation of cell layer GAG which is concomitant with a redistribution of PKC from the membrane to the cytosol. These data, together with the finding that PKC activators reduce GAG sulfation, suggest that in non-differentiated Caco-2 cells which are characterized by the highest level of membrane-bound PKC, the enzyme exerts a negative modulation on this process; this effect may be gradually lost as PKC activity redistributes to the cytosol over the time-course of spontaneous differentiation.

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Thyroxine-induced redistribution of creatine kinase isoenzymes in rat cardiomyocyte cultures

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Summary. The present study demonstrates a change occurring in the creatine-kinase isoenzyme profile of cardiomyocyte cultures induced by a chronic administration of excessive amounts of thyroid hormones (TH). This change is manifested by an increased level of the CK-BB isoenzyme, generally at the expense of CK-MM isoenzyme. The elevation of CK-BB is probably a result of a specific effect of TH through activation of gene expression, rather than a contribution of an increased number of non-myocardial cells. The implications of these results in the diagnosis of heart failures are discussed.
Key words. Creatine-kinase isoenzymes; cardiomyocyte cultures; thyroid hormones.

Creatine kinase (CK) (EC 2.7.3.2) is present mainly in skeletal muscle, heart and brain, although some of its activity can be detected in other tissue[1-3]. The enzyme has a dimeric structure, composed of M or B subunits. Brain CK consists of BB isoenzyme (CK-BB). Adult skeletal muscle consists almost entirely of MM isoenzyme (CK-MM), while cardiac tissue contains, in addition to MM, also some of the MB hybrid form (about 30%). An additional mitochondrial CK (mCK) isoenzyme has been demonstrated[4]. The physiological role of these CK isoenzymes in cellular metabolism is not yet clearly understood. However, it has been noted that in the muscles of long-distance runners, as well as in chronic overloaded myocardium, the level of CK-MB and CK-BB activities was significantly increased[5-9]. The increase has been interpreted as an adaptation to stress.

Since it is documented that hyperthyroidism is associated with a marked increase in heart activity[10,11], our present research was undertaken in an attempt to explore whether an excess of thyroid hormones (TH) could influence the CK-isoenzyme distribution in cardiomyocytes grow in cell cultures, and give rise to effects similar to those of increased use.

Materials and methods

Cell culture. 1–2-day-old rat hearts were removed under sterile conditions and washed 3 times in phosphate buffered saline (PBS) to remove the excess of blood cells. The hearts were minced to small fragments and then gently agitated in proteolytic enzyme-RDB (Ness-Ziona, Israel), which is prepared from a fig tree extract. The RDB was diluted 1:50 in PBS, at 37 °C, for a few cycles of 10 min each. The supernatant suspensions containing dissociated cells, to which medium containing 10% horse serum (HS) was added, were centrifuged at 150 × g for 5 min. After centrifugation, the supernatant phase was discarded and cells were resuspended in high glucose (5 mg/ml) Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated HS and 2% chick embryo extract. The suspension of cells was diluted to 1.6×10^6 cells/ml and 1.5 ml was placed in 32-mm plastic culture dishes coated with collagen-gelatine. Cultures were incubated in a humidified 10% CO_2, 90% air, at 37 °C. Confluent monolayers which exhibit 60–80% of spontaneous synchronous contractions developed in 2–3 days in culture. The growth medium was replaced every 2–3 days. Some cultures were γ-irradiated with 1500 R in order to prevent cell replication. This treatment enriched myocardial cells without inhibiting the spontaneous beatings.

Hormonal treatment. Thyroxine (T_4) or triiodothyronine (T_3) (Sigma) were dissolved in 0.05N NaOH and applied to 4-day-old (or as specified) heart monolayer culture dishes to a final concentration of 1 μM for T_4 or 0.1 μM for T_3. The level of T_4 and T_3 in the serum were 1.5×10^{-12} M and 3×10^{-13} M, respectively.

Creatine kinase (CK) activity and isoenzyme distribution. Culture dishes were rinsed with cold PBS, scraped into 1 ml of 0.1% triton X-100 in NaP 100 mM, β-mercaptoethanol 2 mM pH 7.0. The cells were homogenized at 4 °C and centrifuged 5000 × g for 5 min. Aliquots of the supernatant were measured for total CK activity by the method of Shainberg et al.[12]. Distribution of CK isoenzymes was determined following electrophoretic separation on cellulose acetate using the Helena Laboratories procedure (300 V, 10 min, 25 °C) and equipment. The stripes were then allowed to react with a CK kit and the NADH produced by each isoenzyme band was measured fluorometrically by Helena Labs scanner type Quick Quant II T[13].

Protein synthesis was determined by exposure of the cells (groups of three replicates) to [^{3}H]leucine for 1 h, after which the cells were harvested, homogenized and treated with trichloroacetic acid (TCA). The TCA precipitates were washed, dissolved in 1 N NaOH and counted in a liquid scintillator counter. Protein was measured by the Lowry method.

Results

Exposure of myocardial cultures to 1 μM thyroxine (T_4) induced a 30–70% increase in the rate of cell beating for a few days as measured by direct observation under the microscope. This chronotrophic effect of T_4 was not accompanied by a significant change in CK activity, total protein content or the rate of protein synthesis (table 1). However, there was a transition in the CK-isoenzyme profile following T_4 treatment. The CK-MM isoenzyme decreased by about 20% within 3–4 days, whereas CK-BB was increased by approximately 40% (fig. 1). Similar results were obtained with T_3 treatment (fig. 2). It should be noted that the normal profile of the isoenzyme in untreated cultures was also changed as a function of time: while in young cultures 4–6 days old, the CK-MM is the predominant isoenzyme (around 50%) and BB is less than 20%, in prolonged incubated myocardial cells (longer than 10 days) the CK-BB becomes the major isoenzyme, at the expense of MM. This tendency could be reduced if the cell were γ-irradiated (1500 R) (table 2).

Discussion

In the present study, we have shown that TH induced a change in the profile of CK isoenzymes by increasing the CK-BB at the expense of CK-MM. It is possible that the increase in CK-BB could be a result of increased replica-

Table 1. Effect of T_4 on myocardial cell cultures

	Control	T_4
Beating rate (beats/min)	110 ± 22	170 ± 31
Protein (mg/dish)	1.14 ± 0.25	0.99 ± 0.09
Protein synthesis (dpm/dish)	1941 ± 324	1821 ± 245
CK (mU/dish)	522 ± 112	504 ± 147

T_4 (1 μM) was given to 5-day-old myocardial cell cultures and the measurements were done 48 h later.

Table 2. Effect of T_4 on CK activity

	CK (mU/plate)	Protein (mg/plate)	Creatine kinase (%) MM	MB	BB	mCK
7 DIV control	602	0.71	45	31	24	–
7 DIV T_4 3 days	620	0.74	39	30	31	–
7 DIV irradiated	443	0.55	49	34	17	–
7 DIV irradiated T_4 3 days	456	0.58	42	33	25	–
14 DIV control	1290	1.06	16	30	46	8
14 DIV T_4 3 days	1320	1.11	10	25	60	5
14 DIV irradiated	790	0.67	31	26	37	6
14 DIV irradiated T_4 3 days	785	0.65	22	20	52	6

Total CK activity and percentage of CK isozymes, following electrophoretic separation, from myocardial cells kept 7 and 14 days in vitro (DIV) of treated with T_4. The irradiation (1500R) was performed on the 3rd day in culture.

tion of non-myocardial cells, which have been shown to contain mainly CK-BB[4, 13]. However, this possibility is unlikely, since T_4 also induced elevation in CK-BB isoenzyme in irradiated heart cells and a further decrease in activity of CK-MM. It is possible that TH exert their effect directly through activating the gene for CK-BB. A more likely possibility is that TH activates the heart to contract (overload), as was shown by Kim and Smith in chick myocardial cell cultures[14]. This possibility is consistent with the findings of others, who showed that chronic overloading in vivo modified the CK isoenzyme pattern of cardiac tissue, by increasing the MB and BB forms at the expense of the MM form[8, 9].

Other shifts in relative abundance of isozymes have been reported, under conditions of chronic heart overloading, or in a hyperthyroid state[15]. The most extensively studied is that of myosin in vivo[17, 16] or in myocardial cell culture treated with an excess of T_4[18]. A comparable modification of lactate dehydrogenase isoform has also been reported[19]. It is of interest to note that in all these examples, an isoenzyme that normally almost disappears in the heart reappears during the course of overloading or in the hyperthyroid state. Isomyosin V_3 and the BB-creatine kinase, which appear following overloading, are normally the major isoforms in the fetus. Thus, it is possible that the cardiac myocyte utilizes its inactive fetal capacities in order to improve its efficiency.

Since the K_m values for the formation of ATP from creatine phosphate are smaller for CK-BB than for CK-MM[20], the increased CK-BB activities in thyroid hormone treated cells might facilitate energy transport in stress conditions and be beneficial for the myocardial cells.

Our results may have a clinical implication. Analysis of CK isoenzymes is used as a standard method for diagnosing myocardial infarctions; since we have shown that TH causes alterations in the ratios of the CK isoenzymes, these changes might be a source of error in evaluating

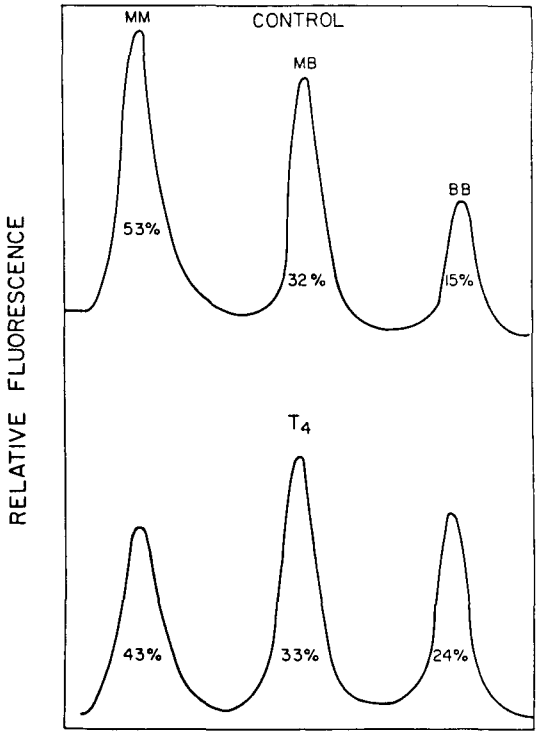

Figure 1. Electrophoretic separation of CK isoenzymes from 7-day-old rat myocardial cultures: control or treated for 4 days with (1 μM) T_4.

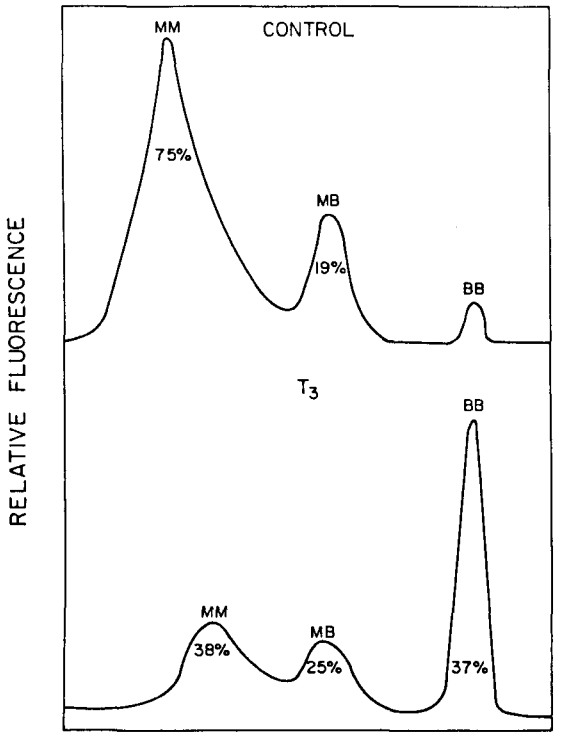

Figure 2. Electrophoretic separation of CK isoenzymes from 8-day-old rat myocardial cultures: control or treated for 6 days with (0.1 μM) T_3.

myocardial damage in hyperthyroid patients. Indeed, it has been shown that CK-BB in the serum of about 50% of the patients suffering from thyrotoxicosis, declined following medical treatment [21]. Thus there is a potential for diagnostic confusion when CK isoenzyme profiles are used.

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Distinction of influenza viruses of different host cell origin

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Summary. Influenza A viruses grown in different animal or human cells retain their antigenic make-up as tested by the usual immunological assays. With the aid of a *Sambucus nigra* (L.) extract containing its lectins the viruses can be distinguished after one single passage in a different cell type by a change in their hemagglutinating properties. Binding of such lectins to influenza viruses may be a means for a more subtle classification, relating to the host cell origin of the virus.
Key words. Sambucus nigra (L.); influenza virus; lectin.

Influenza A viruses grown in different cell types retain their antigenic make-up. The hemagglutinin and the neuraminidase, the two main surface antigens, remain of the same type. Antigenic shifts or drifts which occur in vivo can only be mimicked in vitro by growing the virus in antibody-containing media [1]. Subtle differences which are due to different cellular origins, such as changes in the sugar composition of the hemagglutinin may, however, not be detected by routine immunological methods because of the weak immunogenicity of sugars. By means of a *Sambucus nigra* (L.) extract containing several lectins [2] it is demonstrated that the hemagglutinin of an influenza A virus may vary, depending on the cellular origin.

Materials and methods

Sambucus nigra (L.) extracts. The ripe fruits were collected in northern Switzerland during the second half of September. Ten grams of fruit were frozen in 50-ml Falcon tubes (Falcon Plastics, Oxnard, CA) at − 20 °C. After thawing, the specimens were mashed in the tubes, with a glass homogenizer, and Eagle's minimum essential medium (MEM) was added to a volume of 20 ml. The samples were frozen at − 80 °C. The thawing was then done on a vortex at maximum speed in order that the lumps of ice would homogenize the fruits further. After centrifugation at 2250 × g for 30 min at 4 °C the supernatants were stored at − 80 °C in aliquots of 1 ml until used.

Virus. Avian influenza virus (AIV; A/Turkey/England/63, Hav1 Nav3, Langham strain) had been adapted to primary cultures and cell lines of chicken, mouse, and human origin as described earlier in detail [3–7].

Hemagglutinin determinations. The hemagglutinin titrations were effected using WHO standard procedures [6].

Hemagglutination reduction assay. AIV grown in different cells were mixed with an equal volume of different dilutions of *S. nigra* extract or dilution medium (MEM