and after the incubation period by GLC[8]. Protein was determined by the method of Lowry et al.[12] with bovine serum albumin as standard.

Results and discussion. Of the 3 common plant sterols studied, campesterol was esterified the most, β-sitosterol was next and stigmasterol was esterified very little irrespective of the type of fatty acid present in the incubation medium (table). The esterification of both campesterol and sitosterol was higher with unsaturated fatty acids (oleic and linoleic acids) than with saturated fatty acids. Cholesterol esterification also showed a similar trend. For example, with palmitic acid, cholesterol esterification was only 6.7 nmoles/mg protein/h as compared to about 80 nmoles/mg protein/h when linoleic acid was present in the medium. Since cholesterol, campesterol and sitosterol showed increased esterification with unsaturated fatty acids as compared with saturated fatty acids, the present work is in support of the view that unsaturated fatty acids probably enhance sterol absorption.

In rats, the absorption of sitosterol, measured by radioactive sitosterol feeding, has been reported between 5 and 32%[5]. No information is available regarding the absorption of campesterol and stigmasterol in this species. Based on the present study, it is suggested that in the rat, the absorption of campesterol would be higher than either sitosterol or stigmasterol. Perhaps stigmasterol would not be absorbed. In rabbits fed 2% plant sterols for 10 weeks, it was concluded based on the plasma sterol concentrations that campesterol absorption was greater than sitosterol and probably stigmasterol was not absorbed[13]. Greater absorption of campesterol than sitosterol has also been reported in other species[14–20].

In the newly described lipid storage disease 'β-sitosterolemia and xanthomatosis' involving plant sterols, the absorption of β-sitosterol was very high (about 30% of the radioactive dose fed)[10]. However, campesterol was also found, although in less amount than sitosterol, in the blood and tissues of the patients thereby suggesting absorption of this sterol in amounts less than sitosterol. Both rat and rabbit absorb campesterol in much greater amount than sitosterol. Thus, both these species of animals could not be used as a model for studying the etiology of the newly described lipid storage disease 'β-sitosterolemia and xanthomatosis'.

1 Acknowledgments. Financed by the Emery Goff Research Award from the American Heart Association, Louisiana, Inc. and the USPHS research grant HL-08974 from the National Institutes of Health, Bethesda, Md.

2 L.F. Fieser and M. Fieser, in: Steroids, p.341. Reinhold Publishing Corp., New York 1959.
3 R.G. Gould, R.J. Jones, G.V. LeRoy, R.W. Wissler and C.B. Taylor, Metabolism *18*, 652 (1969).
4 G. Salen, E.H. Ahrens, Jr, and S.M. Grundy, J. clin. Invest. *49*, 952 (1970).
5 M.T.R. Subbiah, Am. J. clin. Nutr. *26*, 219 (1973).
6 A. Kuksis, L. Marai, J.J. Myher and K. Geher, Lipids *11*, 581 (1976).
7 W.E. Connor, J. Am. diet. Ass. *52*, 202 (1968).
8 A.K. Bhattacharyya and W.E. Connor, J. clin. Invest. *53*, 1033 (1974).
9 R.S. Shulman, A.K. Bhattacharyya, W.E. Connor and D.S. Fredrickson, New Engl. J. Med. *294*, 481 (1976).
10 A.K. Bhattacharyya and W.E. Connor, in: The Metabolic Basis of Inherited Disease, 4th edn, p.656. Ed. J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. McGraw-Hill, New York 1978.
11 W.W. Umbreit, R.H. Burris and J.F. Stauffer, in: Manometric and Biochemical Techniques, 5th edn, p.147. Burgess Publishing Co., Minneapolis 1972.
12 O.H. Lowry, N.J. Rosebrough, A.L. Fark and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
13 A.K. Bhattacharyya, Fed. Proc. *37*, 836 (abstr.) (1978).
14 A. Kuksis and T.C. Huang, Can. J. Biochem. Physiol. *40*, 1493 (1962).
15 K.N. Boorman and H. Fisher, Br. J. Nutr. *20*, 689 (1966).
16 M.T.R. Subbiah, B.A. Kottke and I.A. Carlo, Biochim. biophys. Acta *249*, 643 (1971).
17 M.T.R. Subbiah and B.A. Kottke, Experientia *28*, 1431 (1972).
18 W.R. Nes, N.S. Thampi and J.T. Lin, Cancer Res. *32*, 1264 (1972).
19 M.J. Thompson, J.N. Kaplanis, W.E. Robbins and J.A. Svoboda, Adv. Lipid Res. *11*, 219 (1973).
20 M.T.R. Subbiah, B.A. Kottke, I.A. Carlo and M.C. Naylor, Nutr. Metab. *18*, 23 (1975).

Isometric relaxation in rat myocardium: Load dependence and influence of caffeine

C. Poggesi, L. Ricciardi, C. Reggiani and R. Minelli

Institute of Human Physiology, University of Pavia, via Forlanini 6, I-27100 Pavia (Italy), 5 April 1979

Summary. When caffeine plus calcium is added to the perfusing medium, isometric relaxation of rat myocardium is no longer affected by length changes occurring during the twitch. The dependence of isometric relaxation on the initial muscle length is still present and more pronounced after caffeine addition.

In mammalian myocardium[1–3] and skeletal muscle[4,5], isometric relaxation is influenced by displacements or load changes, occurring during the twitch. On the contrary, relaxation in smooth muscle[6] and frog heart[1] is not dependent on the load. The observed difference between these tissues may be related[1] to their different rate of Ca^{2+} removal during relaxation. The high sequestering activity of the sarcoplasmic reticulum (SR) of mammalian heart and skeletal muscle determines an early decline of intrasarcomeric [Ca^{2+}]; cross-bridges cannot further recycle and, if a change in length or load occurs, tension decay depends on the ratio between the number of attached cross-bridges and the load applied.

Because of the feeble action of SR in smooth muscle and frog heart, Ca^{2+} decline is slow: cross-bridges can be reformed and load changes cannot affect the tension fall: in these tissues relaxation depends only on the activation level.

The aim of the present study is to investigate whether, in rat myocardium, caffeine, which has been shown to impair Ca^{2+} uptake by SR[7,8] modifies the dependence of relaxation on load or length changes. In order to describe quantitatively the course of isometric relaxation, it should be useful to fit the curve of tension fall with a mathematical function. The fact that, in rat myocardium, as in skeletal muscle[4,5], tension decay becomes exponential, offers such

an opportunity. The exponential phase of isometric relaxation, evident as a straight line on the phase-plane plot (dT/dt against t) and on the semilogarithmic plot (lnT against t), begins when active tension has fallen to 30–50% of its peak value.

Methods. The experiments have been carried out on isolated papillary muscles from the left ventricles of 2-month-old, male Wistar rats. The criteria for selection of preparations were: 1. Cross-sectional area at L_{max} (length at which tension output is maximum) less than 1 mm^2[2,9]. 2. Resting-developed tension ratio at L_{max} less than 0.25[10]. The muscles were mounted in a thermoregulated bath (24 °C), containing Krebs-bicarbonate solution, bubbled with a gas mixture of 95% O_2 5% CO_2 (pH = 7.45). The preparations, tied by means of silk threads to an isometric force transducer and to a light isotonic lever, were stimulated by supramaximal pulses of 3-msec duration at a frequency of 2/min. In each experiment, isometric twitches at different initial length and isotonic contractions (at L_{max}) with increasing afterload were recorded before and after the inotropic intervention.

In order to obtain the required effect on the relaxation phase and prevent the negative inotropic effect of caffeine on rat myocardium[11] caffeine (10 mM) plus Ca^{2+} (10 mM) was added to the perfusing medium. Tension and length signals were displayed on a storage oscilloscope and stored on a tape recorder. Data processing was carried out by means of a 2-channel memory unit which fed both an XY/t recorder and an alphanumeric printer device.

Each contraction was characterized by the measurement of resting tension (RT), peak developed tension (DT), time to peak tension (tPT), amount of shortening (ΔL) and initial muscle length (L).

A least square regression, performed on the semilogarithmic plot, in the region of exponential tension decline, allowed us to obtain the parameters of the equation $\ln T = -at + b$, corresponding to $T = \exp(-at + b)$.

By means of this approach, we evaluated 2 parameters: 1. the time constant $\tau = 1/a$ (index of the rate of tension decay) and 2. the time when developed tension reduces to 30% of its peak value, $t_{30} = (-\ln(0.3.DT) + b)/a$ (index of duration of the whole twitch, or of relaxation alone when time to peak tension is subtracted: $t_{30} - tPT = t'_{30}$).

Results and conclusions. As already described[1-3] the relaxation phase, in isotonic afterloaded contractions, is shortened (figure 1, C); t_{30} reduces when active shortening increases (by reducing the afterload, figure 1, B).

In agreement with the findings on skeletal muscle[4], the time constant of the exponential phase, τ, is poorly affected by active shortening (figure 1, A). The opposite observation on cat myocardium[12] might be attributed to the different species studied or to the different representation of results.

Isometric relaxation decelerates and is prolonged by increasing the initial muscle length: τ and t'_{30} are found to be directly related to the initial length (figure 2, A and B). This observation agrees with the results obtained on frog[4] and rat[13] skeletal muscle.

The most striking effect of the addition of caffeine plus Ca^{2+} to the perfusing medium refers to the relaxation phase: τ and t_{30} are strongly increased (table).

The effect of an isotonic phase on the isometric relaxation is completely abolished by caffeine: tension decline, in isometric and afterloaded twitches, largely coincides (figure 1, D).

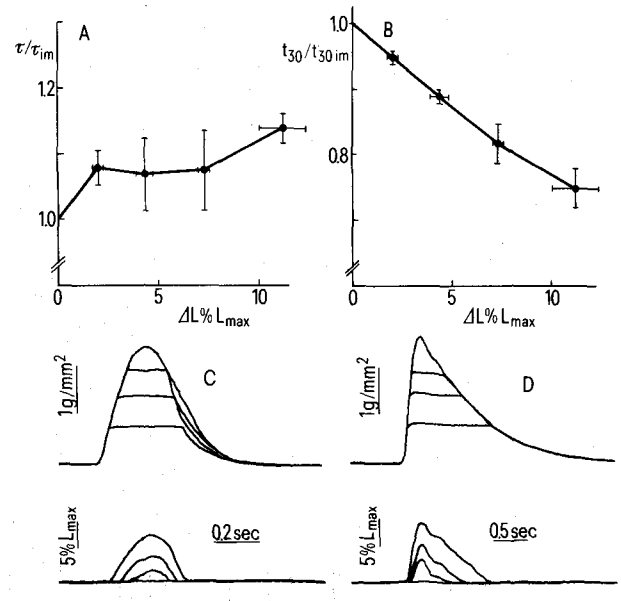

Fig. 1. Isometric relaxation of rat papillary muscle in afterloaded twitches performed at L_{max} and 24 °C. *A* and *B* Relationships between the parameters of the exponential phase of isometric relaxation (τ and t_{30}) and the amount of displacement occurring during the twitch (ΔL). τ and t_{30} are expressed as ratio with the values measured in the complete isometric twitch (τ_{im} and $t_{30\,im}$). ΔL is expressed as % of L_{max}. Means ± SE of 6 experiments are reported. *C* and *D* Isometric and afterloaded twitches in control conditions (C) and after caffeine (10 mM) plus Ca^{2+} (10 mM) addition (D).

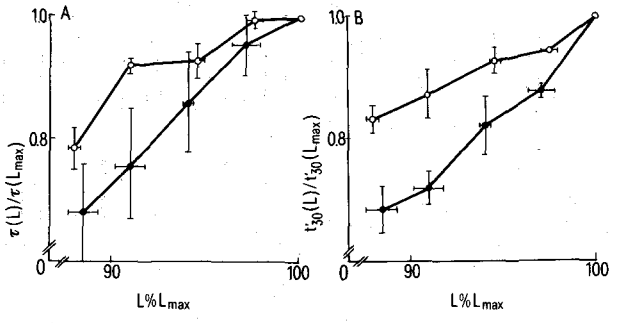

Fig. 2. Effect of the initial muscle length on the parameters of relaxation (τ and t'_{30}) of isometric twitches performed at 24 °C, in control condition (○) and after caffeine (10 mM) plus Ca^{2+} (10 mM) addition (●). t'_{30} is equal to t_{30} - tPT. Initial muscle length (L) is expressed as percent of L_{max}. τ and t'_{30} are expressed as the ratio with their values in the isometric twitch performed at L_{max} ($\tau(L_{max})$), (t'_{30} (L_{max})). Means ± SE of 6 experiments are reported.

Some parameters measured in isometric contractions performed at L_{max} and 24 °C in control condition and after caffeine (10 mM) plus Ca^{2+} (10 mM) addition. Means and SE of 6 experiments

	RT (g/mm^2)	DT (g/mm^2)	tPT (msec)	τ (msec)	t_{30} (msec)
Control	0.75 ± 0.12	3.94 ± 0.75	208.0 ± 10.42	92.38 ± 5.71	524.80 ± 55.02
Caffeine plus Ca^{2+}	0.71 ± 0.09	3.50 ± 0.56	263.8 ± 21.03	793.00 ± 158.70	1436.40 ± 180.60

This might be due to the impairment of the Ca^{2+} uptake ability of SR, caused by caffeine[7,8]; owing to the increased Ca^{2+} availability in the myoplasm, during relaxation, cross-bridges may be reformed, and the load dependence of tension decay disappears. The influence of the initial muscle length on isometric relaxation still remains in the presence of caffeine: the dependence of the rate and duration of isometric relaxation on the initial length seems to be more pronounced after caffeine addition (figure 2, A and B). No clear explanation of this caffeine effect is provided by this investigation.

These observations suggest that, in isometric twitches, performed under both conditions (control and caffeine), the time course of relaxation depends on the activation level (initial muscle length), while the load control of relaxation takes place only when activation has a very early decline.

1 D.L. Brutsaert, N.M. De Clerck, M.A. Goethals and P.R. Housmans, J. Physiol. *283*, 469 (1978).
2 A.J. Brady, Fedn Proc. *24*, 1410 (1965).
3 R.L. Kaufmann, R.M. Bayer and C. Harnasch, Pflügers Arch. *332*, 96 (1972).
4 B.R. Jewell and D.R. Wilkie, J. Physiol. *152*, 30 (1960).
5 A.S. Bahler, Am. J. Physiol. *220*, 1983 (1971).
6 B. Johansson and P. Hellstrand, Acta physiol. scand. *93*, 167 (1975).
7 L. Blayney, H. Thomas, J. Muir and A. Henderson, Circulation Res. *43*, 520 (1978).
8 J.R. Blinks, C.B. Olson, B.R. Jewell and P. Braveny, Circulation Res. *30*, 367 (1972).
9 C.L. Gibbs, Physiol. Rev. *58*, 174 (1978).
10 F.J. Julian and M.R. Sollins, Circulation Res. *37*, 299 (1975).
11 R. Bodem and E.H. Sonnenblick, Am. J. Physiol. *228*, 250 (1975).
12 W.W. Parmley and E.H. Sonnenblick, Am. J. Physiol. *216*, 1084 (1969).
13 D.K. Hill, J. Physiol. *221*, 161 (1972).

Effect of hormones on adenyl cyclase activity of rabbit mammary gland in vitro

J. Singh and N.C. Ganguli[1]

National Dairy Research Institute, Karnal-132001 (India), 20 February 1979

Summary. Ovarian hormones namely β-estradiol and progesterone were observed to stimulate the activity of adenyl cyclase of the mammary gland from pregnant rabbit in vitro, unlike the lactating tissue where it was inhibited. On the other hand, non-ovarian hormones like hydrocortisone, prolactin and insulin did not have a similar effect on this enzyme.

Hormones have been observed to be required for the mammary gland development and its metabolic function[2,3] during pregnancy and lactation. Cyclic AMP has been established to be the second messanger of hormonal stimulation at the cell surface[4]. Adenyl cyclase activity, which is responsible for the synthesis of cyclic AMP was observed to change during lactation cycle[5]. The present investigation was therefore undertaken to note the effect in vitro of hormones, both ovarian and non-ovarian, on the activity of this enzyme in the mammary gland from pregnant and lactating rabbits.

Materials and methods. Animals at various stages were obtained from the small animal house maintained at the institute. β-Estradiol, progesterone, hydrocortisone, caffeine, prolactin and insulin were purchased from Sigma Chemical Company, USA. The other chemicals used were of the analytical grade.

Mammary tissue was collected from rabbits at various stages of lactation and washed with cold 0.1% KCl solution. 5 g tissue was minced for 10 min and homogenized in 20 ml of 50 mM Tris-HCl buffer (pH 7.6). Homogenized tissue was strained through 4 layers of muslin cloth and the extract obtained was spun in a high speed centrifuge at 4000× g for 20 min. The pellet was washed twice with buffer and was resuspended in the 2.0 ml of buffer which was used as enzyme preparation[5].

Adenyl cyclase assay was carried out by the method of White[6] with certain modifications. Assay system contained 50 mM Tris-HCl buffer, pH 7.6, bovine serum albumin, 10 mM mercaptoethanol, 20 mM caffeine and ATP generating system. Final volume of the assay system was 0.15 ml containing 0.25 μCi 8-C^{14}-ATP (0.4 mM), steroid hormones (0.02 mM) and peptide hormones (0.3 units/ml). The enzyme was preincubated for 10 min at 37 °C with steroid hormones and peptide hormones. 50 μl of preincubated enzyme was added to the assay system and incubated for 20 min at 37 °C. Control sample was without the hormone addition. The reaction was terminated by keeping the mixture in boiling water bath for 3 min, cooled to room temperature and 1 ml of imidazole-HCl buffer (pH 7.0) was added.

The rest of the procedure followed was as described earlier[5]. The specific activity of the enzyme was expressed as n mole of cAMP produced/mg protein/min at 37 °C.

Results and discussion. Ovarian hormones have a stimulatory effect on the enzyme isolated from pregnant animal as shown in the Figure. Progesterone and β-estradiol have a synergistic effect on the enzyme preparation from early and mid-pregnant rabbit, where the stimulation caused by the hormones put together in the in vitro system was higher than their individual effect. Whereas such effect was not

Adenyl cyclase of rabbit mammary gland as effected by hormones in vitro

Hormone	Adenyl cyclase specific activity (nmoles/mg protein/min)	
	Late pregnancy (26 days) Stimulation (%)	Late lactation (56 days) Inhibition (%)
β-Estradiol	39.1	15.7
Progesterone	21.3	25.5
β-Estradiol + progesterone	30.2	31.0
Hydrocortisone	68.3	5.9
Prolactin	35.1	25.5
Insulin	-9.8*	43.1

* Inhibition (%).