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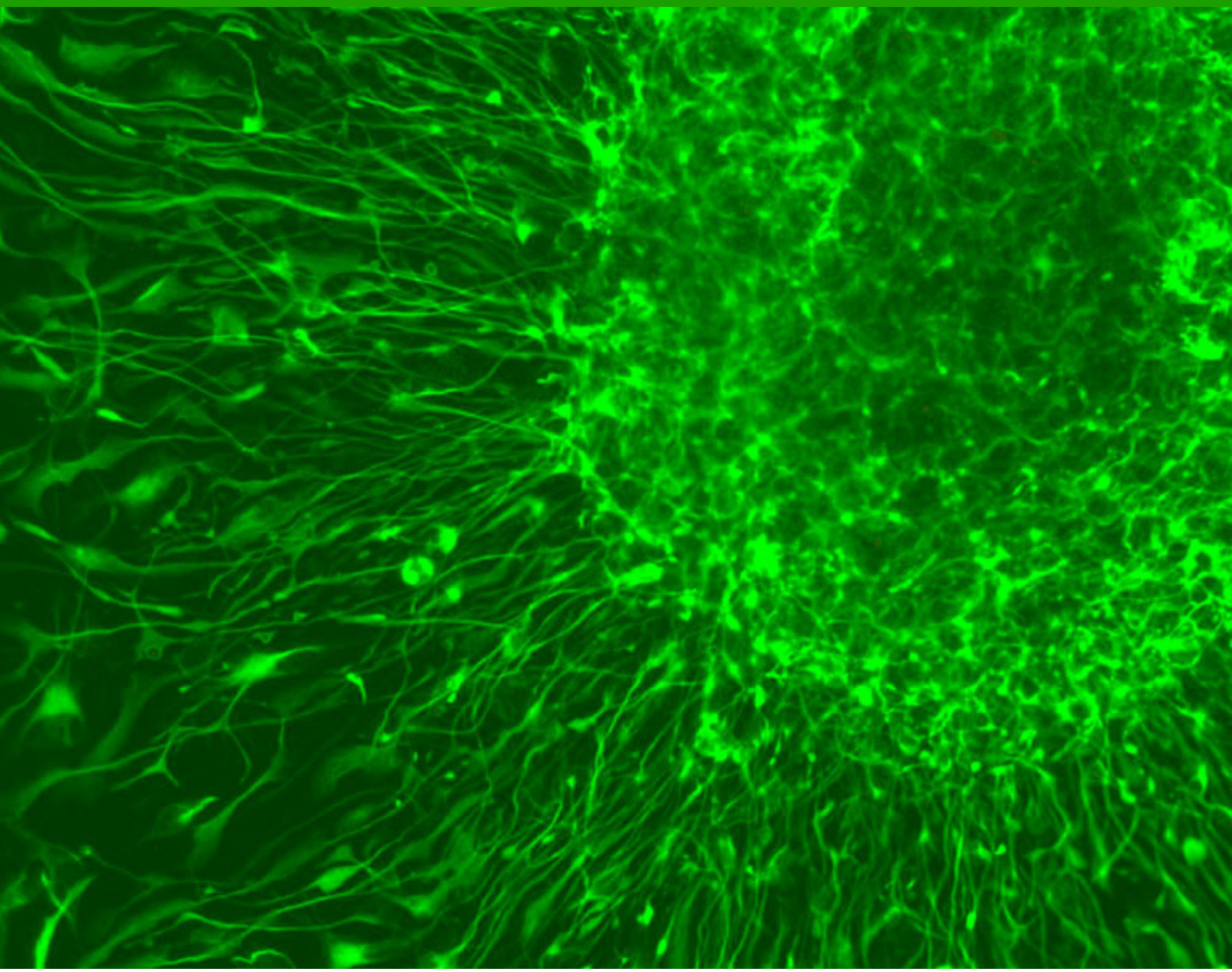

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CALORIMETRY OF ISOLATED HEARTS (I) A METHOD TO STUDY THE ENERGETIC OF Ca^{2+} HOMEOSTASIS DURING PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

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Abstract

Calorimetry has been applied to skeletal and cardiac muscle from many years ago, and there were intents to calculate the heat fractions associated to basal metabolism (resting heat rate, Hr) and the active heat (Ha). This review explains the principles and evolution of cardiac energetic, measured by calorimetry and the advantages with respect to the measurement of oxygen consumption. Moreover, the methods to estimate separately the fractions of tension-dependent (TDH) and tension-independent heat (TIH) were described, as well as the characterization of each one. A method to determine 4 heat components in a single beat in the presence of contraction in perfused rat ventricles was revised, as well as the properties of the long-duration fourth fraction not previously seen with other methods. The advantages and limitations of each method were analyzed. Calorimetry allows to evaluate the occurrence degree of certain exothermic processes such as ionic fluxes can be studied by comparing the heat released with the energetic equivalent and the stoichiometry of the process with the ATP hydrolysis. Calorimetry is a sensitive and useful methodology to evaluate the "in situ" incidence of exothermic mechanisms in the heart.

Keywords: calorimetry, heart, basal metabolism, active heat, TDH and TIH

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1. Cardiac calorimetry: principles and historical evolution

Calorimetry is a physicochemical method that elicits the study of biological systems in terms of energy exchange as heat. The quantity of heat produced and its release kinetics depends mainly on temperature as metabolic processes do and also the external work done as the first law of thermodynamics rises. The myothermal approach to the study of muscle physiology has come a long way since the pioneer studies on skeletal muscle carried out by Helmholz and followed by AV. Hill [1] during the first half of the last century. The difficulties to apply the technical developments achieved so far to cardiac muscle were such harder that only well into the sixties it was possible to make measurements on small pieces of beating cardiac muscle such as papillary muscle of small mammals (rabbits, rats) resting on thermopile device [2]. The specimen utilized should be sufficiently thin so that diffusion of oxygen and nutrients from the superfusion media was appropriate to avoid the establishment of a hypoxic core mainly at the moment of recording heat released, since it was necessary to briefly drain the thermopile to make a measurement [3]. Following the formalism introduced by Hill [4] for skeletal muscle contraction in which the metabolic recovery could be safely separated from the contractile processes at low temperatures, Chapman and Gibbs [5] promoted a model for heat evolution during a muscle twitch (initial heat, IH) as:

$$IH = A + \Delta H \int k \cdot n \, dt \qquad (1)$$

Where: A is the activation heat or tension independent heat (TIH); n: instantaneous number of acto-myosin-cross-bridges links; ΔH: molar enthalpy of ATP hydrolysis; k: constant that quantifies the rate of dissociation of cross-bridge links that depends on species, type of muscle, temperature and inherent ATPase activity.
The second term in the equation (1) corresponds to tension dependent fraction of heat released (TDH) during a twitch. Since in cardiac muscle even at temperatures as low as 20°C most of the recovery heat is released within the period of contraction [6] the heat released during a twitch in a steady train of contractions (better called activity-related heat, Qa) is best described by de sum of two fractions as indicated above, but including the recovery fraction of the chemical energy associated to each one. That is:

$$Qa = TIH + TDH \qquad (2)$$

The TIH fraction has been associated with energy expenditure as heat mainly due to the active transport of sodium and calcium, in addition to binding of Ca^{2+} to troponin (TnC), conformational changes in thin filaments, and oxidative reactions that rephosphorylate ADP. The second term (TDH) encloses the chemical energy of ATP used for acto-myosin interaction to generate force and its recovery mainly by oxidative phosphorylation. TDH could be calculated from the difference between Qa and TIH measured. The evaluation of TIH requires that no acto-myosin interaction exists. Cardiac muscle cannot be stretched beyond its optimal length (Lo, in which active force development is optimal) since irreversible damage would occur. Therefore, TIH fraction was not easily measured like it was in skeletal muscle. Then, there were developed several strategies to achieve this purpose such as preshortening technique [3], the use of hypertonic solutions with mannitol or cardioplegic compounds such as butanedione monooxime (BDM) [7] or the entirely biophysical latency relaxation technique [8]. All this techniques for TIH measurement were developed for thin papillary muscles using thermopile calorimetry. With this technique, absolute values for basal metabolism (ie. the heat released of quiescent muscle)

were difficult to estimate due to the baseline instability. Almost in the nineties, and to overcome the possibility of deficient oxygenation, a flow-through microcalorimetric technique suitable to make measurements of both basal and active metabolism on small trabeculae from guinea pig heart was developed [9]. This technique had the disadvantage of not including simultaneous measurement of the force developed. More recently and based in the flow-through mode, Dr. Loiselle´s group in New Zeeland developed and improved a microcalorimeter that allows to measure both heat and mechanical parameters in small pieces of muscle overcoming the last cited disadvantage [10,11].
At the whole heart level, calorimetry offered safely measurements since the pioneer work of Neill et al [12], who was able to calculate, in *in situ* experiments and a variety of hemodynamic conditions, the heat released from the determination of blood circulation velocity and the detection of temperature differences using termistors placed into the ascending aorta and *venous sinus* of dog heart. The same type of myothermal methodology was applied to isolated heart placed in a Dewar flask and perfused by Langendorff method [13]. From the interventricular pressure, the author estimated the wall stress using a simple spherical model for the heart revealing that about 50% of total heat was tension independent. Later, this technique was improved through the introduction of simultaneous polarografic measurement of oxygen consumption [14]. Somewhat later, it was developed and subsequently improved a calorimetric system that elicits the simultaneous measurements of heat rate of quiescent and beating cardiac muscle join with mechanical force or intraventricular pressure in arterially perfused preparations [15,16]. As physiological perfusion is used, the problem of oxygen restriction is solved. The system also allows collecting the effluent for analysis for lactate production in order to quantify the extension of anaerobic metabolism [15]. In addition, and to highlight, it is the only system that, in our knowledge, allows to continuously study the processes related to global ischemia and reperfusion without appealing to the use of incubation solutions for simulate ischemia [17,18,19,20,21, 22,23,24].
In summary, after this short history about the evolution of myothermic measurements, the view of muscle (cardiac) energetic based in the current consensus [25] can be encapsulated by the following equation (Eq. 3), which takes into account the first law of thermodynamics that at constant pressure and temperature and at negligible volume change implies that the enthalpic change (ΔH) is expressed as:

$$\Delta U \cong \Delta H = Q + W = Q_B + Q_A + Q_F + W \qquad (3)$$

Where: Q_B: heat released under basal (quiescent) conditions
Q_A: activation heat or TIH
Q_F: force dependent heat or TDH
W: external work done, which is zero when muscle contracts isometrically.
ΔU: internal energy

Each one of the indicated heat fractions can be associated with different processes that take place in a functional cardiac tissue.

2. Heat fractions: active and resting heat

As explained, cardiac energetic has been analysed in terms of two basic processes: a) the active metabolism related to the excitation-contraction-relaxation cycle, and b) the resting metabolism [25].

2.1. Active heat

It is possible to measure the total heat flow (Ht) during the steady state stimulation, and the resting heat rate (Hr) during the mechanical resting state, both expressed in $mJ.s^{-1}.g^{-1}$. The energy associated to one cardiac contraction is called "active heat" (Ha, expressed in $mJ.g^{-1}$), and can be calculated as the area under the curves of total heat rate (Ht) and resting heat rate (Hr) between two beats. Practically it can be obtained from the following equation:

$$Ha = \frac{(Ht - Hr)}{HR} \quad \text{where HR is heart rate (in } s^{-1}\text{)} \quad (4)$$

As above explained, in cardiac muscle there is not a separation between TIH, TDH and the recovery heat (RH), because heart is continuously recovering the ATP consumed in order to maintain the periodic cycles of contraction and relaxation. So, both components of the active heat, TDH and TIH, include the respective fraction of recovery heat. There were intents to individually determine the energy fractions as explains below.

Tension-independent heat (TIH)

As previously explained, the different strategies to calculate the heat fractions contained in Ha include the elimination of the contractile component, either by using hyperosmotic solutions, gradual reduction of stretching in steps up to the loss of contractility [3, 25] or quick-release of muscle from its initial length [8]. All of them were used in papillary muscles with the thermopiles method and permitted the determination of the TIH. The gradual shortening and extrapolation to zero-length of the linear regression between total energy (Ht) and muscle length, resulted in TIH values of about 2 $mJ.g^{-1}$ in rat papillary muscles [26]. Mulieri and Alpert [27] used a medium with mannitol at 2.5 times the normal osmolarity in order to minimize the myofilaments interaction. The quick release method consists in releasing an edge of the muscle after about 15 ms from the electrical stimulation; that is, during the latency period so that the contraction does not occur [8]. At physiological $[Ca^{+2}]_o$ the TIH values were also different among the animal species, with values between 0.7 and 3 $mJ.g^{-1}$ wet weight [8,27,28,29]. With the quick-release method the TIH values ranged between 2 and 4 $mJ.g^{-1}$ in rabbit papillary muscles, depending on the $[Ca^{+2}]_o$ [8]. Other determinations of the activation energy were obtained by linearly correlating the oxygen consumption with the pressure-volume area in rabbit or dog working whole hearts, from which the extrapolation to zero-area resulted equivalent to about 4.3 $mJ.g^{-1}$ [30]. In spite of these differences obtained among the different methods, conceptually the TIH must be higher than the respective fraction measured by oxygen consumption, because the heat release detect the aerobic processes and also the exothermic Ca^{2+} binding. The values of TIH were contrasted with biophysical and biochemical measurements of ionic fluxes, showing correspondence or allowing to estimate the real participation *in situ* [27, 31, 32].

Tension-dependent heat

The tension-dependent heat (TDH) was first estimated in rabbit papillary muscles by using the thermopiles method and gradual pre-shortening, from the slope of linear regression between total heat and contractile tension, obtaining values of about 0.17 $mJ.mN^{-1}.mm^2.g^{-1}$ (by quick-release), and 0.22 $mJ.mN^{-1}.mm^2.g^{-1}$ [26] to 0.33 $mJ.mN^{-1}.mm^2.g^{-1}$ [27]. These estimations of TDH include the recovery heat associated to mitochondrial metabolism. So, an alternative was to estimate the tension-dependent energy in whole hearts by calculating the slope of linear correlation between the oxygen consumption and the pressure-volume area [30].

The last method to determine the heat components was proposed by Ponce-Hornos et al. [16] with the use of a flow calorimeter for isolated heart muscles. This method was based in the analysis of a diffusional heat transfer inside the chamber, from the muscle to the Peltier thermal units considering both, the cooling rate and the diffusion delay constants. The function was applied to the Ht vs. time recording during a beat, to adjust several components which differ in their rate constants and sequentially reach their peaks. This analysis was applied to either a single contraction or one beat extracted from a train during the steady-state stimulation in rat ventricles. A difference from other methods as the thermopiles, in this method it was possible to simultaneously define four components in the presence of force, and consequently calculate the energy associated to each one (Figure 1). In the isovolumic contractions there were fitted two components with the lower time-to-peak, called H1 and H2. Both fractions were independent on the pressure development (TIH) and dependent on Ca^{2+}, but only the second (H2) was increased by a twin stimulus added after a brief period in which TnC was still saturated by Ca^{2+} [16]. Because of these, H1 was associated with the binding of Ca^{2+} to TnC, and H2 was related to the active removal of Ca^{2+}. This origin of H2 was also confirmed in another work [33]. The third component H3 has characteristics of TDH and contains the heat fraction associated to the aerobic metabolism for ATP recovery, since the slope of the H3 vs. P correlation was decreased by bubbling nitrogen [16]. The forth fraction (H4) was the more sensitive to hypoxia, it was independent on the contractile tension developed, and evolved slowly as a steady heat rate during about 100 sec before turning off in the single beat. In the subsequent 2 or 3 beats H4 grew up to become a suprabasal heat rate component [16]. The H4 fraction has never been described by the thermopiles method, because in this one it is not possible to continue the measurement for more than about 30 sec [27]. Under cardioplegic solution (25 mM K-0.5 mM Ca Krebs) the H4 component of the single beat showed a higher sensitivity to hypoxia than P or the other heat components [34]. Moreover, under cardioplegia H4 strongly raised with $[Ca^{2+}]_o$ and was sensitive to the Ca^{2+}-channel blocker verapamil. Both Ca^{2+}-dependence and verapamil sensitivity of H4 were bigger than those of P, TDH (H3) or TIH (H1 and H2), respectively. All these characteristics allowed us to suggest that H4 would represent a metabolic activation related to a Ca^{2+} cycling through the mitochondrial membrane. Figure 2 shows a schematic representation of the main exothermic events associated to ionic movements, contractility and resting or active metabolism in a cardiomyocyte, with the corresponding heat fraction. We have also showed that high $[K^+]$-cardioplegia induces depletion of the sarcoplasmic reticulum (SR) Ca^{2+} storage, reduces contractility and slows the last period of relaxation, so remaining more cytosolic free Ca^{2+} available to mitochondria [34]. Although the mitochondrial Ca^{2+} transporters were described at that time in heart [35], their known function was to regulate metabolism and trigger a dysfunction upon overload. In the ninety´s, it was not still attributed a role to the mitochondrial Ca^{2+} transporters (uniporter and mNCX) in the contractile cycle, such as it was found in the following years when measuring the oscillations of $[Ca^{2+}]_m$ driven by the cytosolic Ca^{2+} transients [36,37]. It was an aim of our

following calorimetrical studies to find the mitochondrial role in the models of cardiac stunning induced by ischemia and reperfusion. So, we first described that after no-flow ischemia single beats developed at intervals of 1 minute lost quickly the H4 component. Moreover, the reduction of the first 2 components agree with the fall in Ca^{2+} influx induced by the hypoxic condition and the fall of H3 agree with the fall in P [17]. Nevertheless, new findings about the mechanism of stunning under reperfusion had to be done under conditions of stimulation nearer to the physiological one that is at least 3 Hz of stimulation and 37°C, in which it is not possible to obtain the individual signal of heat rate or their components. Consequently, the following calorimetrical studies about the energetic of the stunning due to ischemia and reperfusion were done in hearts stimulated under those conditions of frequency and temperature, with the measurement of the steady total heat rate (Ht).

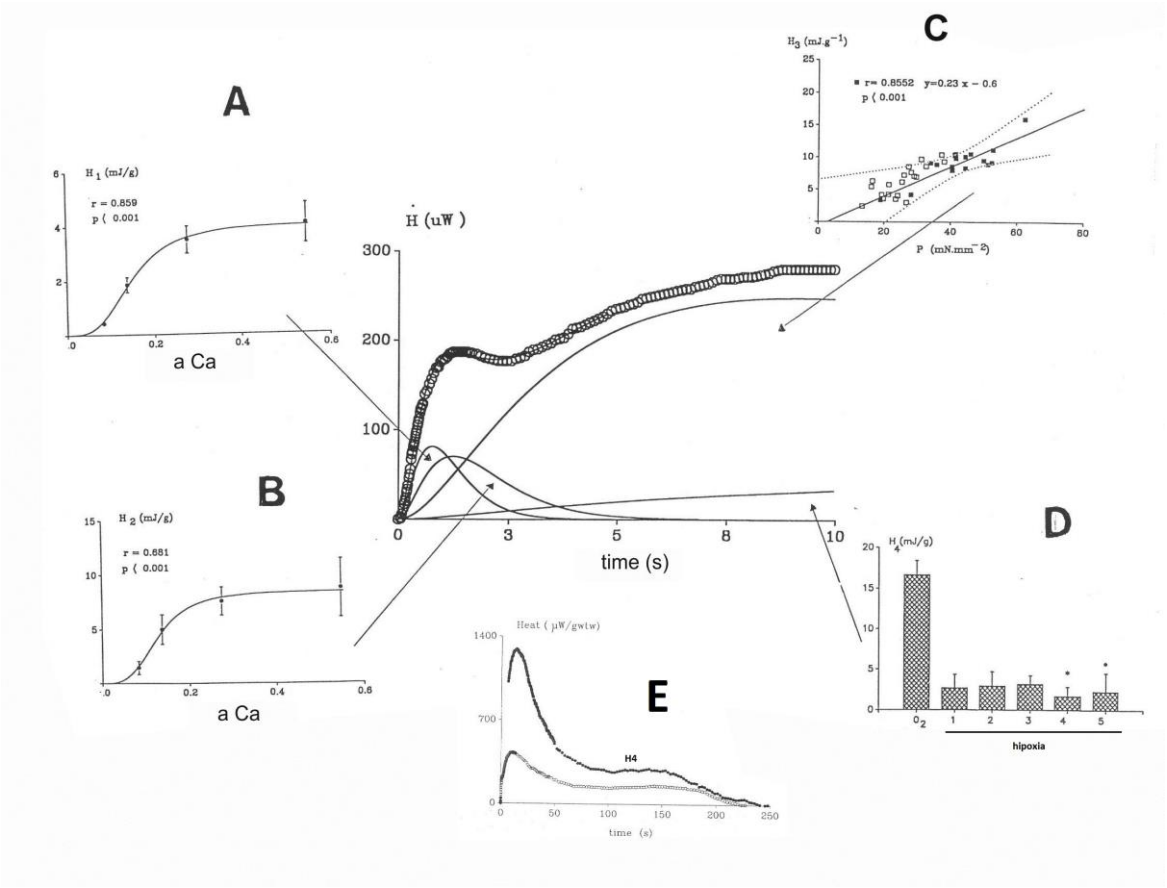

Figure 1: Typical recording of the heat production (Ht in µW vs. time) from a single beat obtained at 25°C in a perfused rat heart, and the four components fitted by using the difussional function of the calorimeter. The arrows show the main characteristical evidences of each heat fraction: Ca-dependence of H1 (A) and H2 (B), pressure-dependence of H3 (C) and sensitivity to hypoxia (95% N_2 bubbling) of H4 (D). Pannel E shows the Ht recording of 1 and 3 beats, with the indication of H4 as the long-lasting steady component. For a complete description see [16].

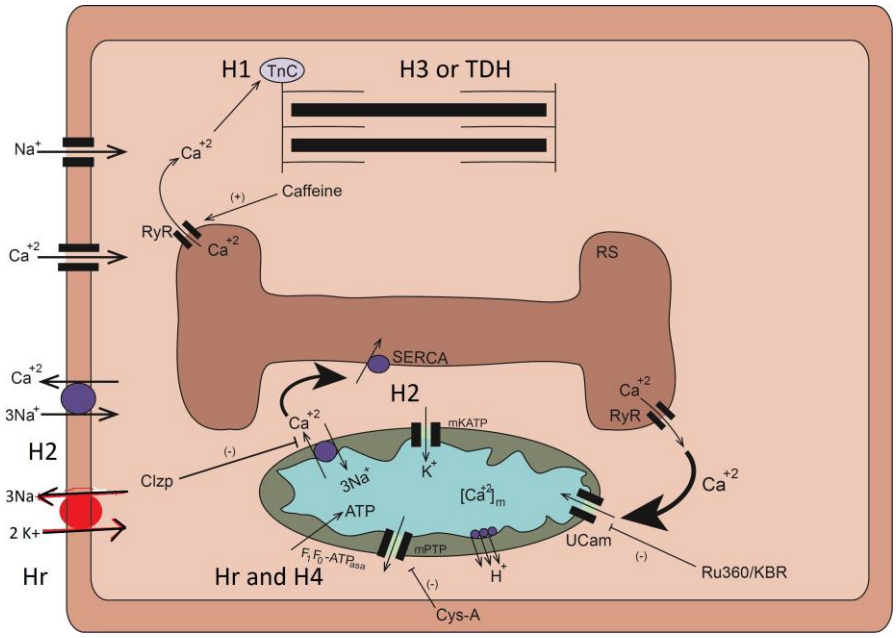

Figure 2: Schematic representation of the main exothermic events associated to ionic movements, contractility and resting or active metabolism in a cardiomyocyte, with the corresponding heat fraction (Hr: resting heat rate, TDH: tension dependent heat, H1, H2, H3 and H4: heat fractions described on the text).

2.2. Resting heat

The resting metabolism has been estimated by measurements of oxygen consumption or calorimetry by rendering the heart inactive, either by eliminating the spontaneous activity or by perfusing a cardioplegic solution of high $[K^+]_o$. The first estimations of resting heat rate (Hr) were obtained by Loiselle and Gibbs [28]. It was reported that both, Hr and O_2 consumption under resting conditions were independent on temperature [38, 39]. Contrarily, Hr increased with the preload or muscle length [2] and was dependent on both, metabolic substrate and animal species [40, 41]. The metabolic substrate is one of the determinants of resting heat release, since it is a trigger of different metabolic paths. Chapman and Gibbs [42] described that in rabbit papillary muscles Hr is maximal for pyruvate (Pyr, about 3 $mW.g^{-1}$ wet weight) which is an exclusive aerobic substrate that induces acidosis, and enters to the myocyte and mitochondria through a symporter with H^+ with the energy consumption [43]. For other substrates, Hr was decreasing in the order of acetate (about 2.7 $mW.g^{-1}$), lactate (about 2.3 $mW.g^{-1}$) and glucose (about 1.8 $mW.g^{-1}$) [42].

The $[Ca^{2+}]_o$ has not influence on Hr contrarily to the strong effect that it has on the total heat rate (Ht) due to the Ca^{2+}-dependence of contractility [29]. However, extracellular Ca^{2+} withdrawal induced an increase in basal metabolism dependent on the intracellular rise in both, Na^+ and Ca^{2+} [44]. Different is the influence of $[Na^+]_o$ which contributes to regulate the activity of the Na, K-ATPase, since it was found a linear correlation between Hr and the $[Na^+]_o^2$ in the rabbit cardiac septum [45]. Under physiological $[Na^+]_o$ the energy consumed by the Na^+ pump was calculated to represent about 12 to 27 % of Hr [45]. Part of the Na, K-ATPase activity is related to the coupling with the sarcolemmal Na/Ca exchanger (SL-NCX), and was calculated that at resting $[Ca^{2+}]_i$ of 0.05 μM its energy consumption represents about 0.14 to 0.29 $mW.g^{-1}$ [31]. The rest of Hr (about 70%) was also related to oxygen consumption, and sensitive to hypoxia, by which it was interpreted as the energy associated

to basal mitochondrial metabolism [46]. The mechanisms of Ca^{2+} homeostasis increased the energy consumption under a high-$[K^+]_o$ cardioplegia [34, 46].
In conclusion, this first review shows the principles and advantages of cardiac calorimetry, concluding that it is a traditional, very sensitive and useful methodology. The following second review of cardiac calorimetry will show some recent findings related to the application in the study of the underlying mechanisms of resting heat rate and the stunning induced by ischemia/reperfusion.

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