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Some observations on Ca-overload in rat ventricular tissue

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It is well known that Ca-antagonists are able to protect myocardium, arterial wall and other tissue from deleterious Ca uptake. This has been shown using atomic absorption spectroscopy as far as the *total* Ca-content of the tissue is concerned. The behaviour of *free* intracellular Ca in this context has, however, remained unknown so far. In our experiments myocardial Ca-overload has been induced by a single high dose of vit. D_3. Here we show that vitamin D_3-induced myocardial Ca-overload can be prevented by diltiazem. Using Ca^{++}-selective microelectrodes in Sprague-Dawley rats we demonstrate that a single high dose of vit. D_3 led to an excessive rise in intracellular *free* resting calcium ($(Ca^{++})_i$). This myocardial $(Ca^{++})_i$-overload has been effectively prevented by s.c. diltiazem (300 mg/kg/d) and verapamil (60 mg/kg/d). Thus, we found resting values of myocardial $(Ca^{++})_i$ of $2.0 \pm 0.2 \times 10^{-7}$ mol/l (n = 7). In vit. D_3 treated rats $(Ca^{++})_i$ amounted to $3.2 \pm 1.0 \times 10^{-5}$ mol/l (n = 7). Conversely, in the vit. D_3 + diltiazem and vit. D_3 + verapamil groups $(Ca^{++})_i$ was $1.6 \pm 0.8 \times 10^{-6}$ mol/l and $2.1 \pm 0.7 \times 10^{-6}$ mol/l, respectively.

We conclude that Ca^{++}-antagonists can prevent the vit. D_3-induced excessive rise in *myocardial free* intracellular Ca^{++}-concentrations. In addition, we show that Ca^{++}-selective microelectrodes constitute a suitable means for studying the effect of pharmacological agents on $(Ca^{++})_i$. *J Clin Basic Cardiol 1999; 2: 255–8.*

Key words: myocardium, rat, Ca-overload, microelectrode, Ca-antagonist

The importance of intracellular free calcium for various cellular functions is well known. For example, changes in intracellular free calcium ($(Ca^{++})_i$) play a key role in the regulation of muscle contraction, neurotransmitter release, glandular secretion and cell to cell communication [1, 2]. Because of its importance, $(Ca^{++})_i$ is controlled at several different levels: sarcolemmal, mitochondrial and via the sarcoplasmic reticulum [3]. These different regulatory mechanisms maintain $(Ca^{++})_i$ at a constant and very low resting level. For example, in ferret myocardial cells $(Ca^{++})_i$ was measured to be 2.6×10^{-7} mol/l [9], which is 10 times less than Ca^{++} measured by us in normal heat-distilled water (Table 1). Thus, cellular Ca-overload exerts a deleterious effect on cell function and can lead to cell death [4]. Intracellular Ca-overload has, for instance, been observed during ischaemia in myocardial tissue [4]. Ca-overload can also be produced by a single high dose of vit. D_3 [5]. It has been shown in this context that Ca-antagonists effectively prevent vit. D_3-induced myocardial Ca-overload [5, 6]. Using atomic absorption spectroscopy (AAS), electron microscopy and histological techniques, the *total* myocardial tissue content of Ca has been studied in vit. D_3-treated rats [eg, 6]. With these methods, however, it is not possible to demonstrate whether or not *free* intracellular Ca^{++} increases along with bound Ca and to what extent this may happen. In the present study we measured $(Ca^{++})_i$ with ion-selective microelectrodes in papillary muscles of rats after they had received a high dose of vit. D_3 in the presence and absence of the Ca-antagonists diltiazem and verapamil.

Table 1. Values of myocardial $(Ca^{++})_i$ measured by different authors using different techniques.

Animal	(Ca^{++})	Method	Author*
Sheep	3.6×10^{-7}	Ca^{++}-electrode	Corey et al.
Ferret	2.6×10^{-7}	Ca^{++}-electrode	Morban et al.
Rabbit	2.7×10^{-7}	Ca^{++}-electrode	Lee et al.
Guinea pig	2.9×10^{-7}	Ca^{++}-electrode	Corey et McGuigan
Rat	2.0×10^{-7}	Ca^{++}-electrode	present study
Rat	1.8×10^{-7}	aequorin	Cobbold et Bourne
Rat	1.81×10^{-7}	quin 2	Sheu et al.

*Literature can be obtained from authors

Material and methods

Vit. D_3 (Vi-De 3 Hydrosol, Wander AG, Bern) was administered to Sprague-Dawley rats weighing 450–550 g i.m. at a single dose of 250,000 IU/kg body weight. Diltiazem and verapamil were given i.m. at a dose of 300 mg/kg/d and 60 mg/kg/d respectively. After three days the rats were put asleep by a heavy blow on their head, their hearts were quickly removed and placed in oxygenated Tyrode solution. The right ventricle was opened, a suitable papillary muscle removed and then attached to a force transducer. The latter then was placed in an experimental chamber superfused with Tyrode solution (37 °C). The Tyrode solution contained (in mmol/l): 131 NaCl, 4 KCl, 2 $CaCl_2$, 1.05 $MgCl_2$, 5 glucose, 0.41 NaH_2PO_4 and 13 $NaHCO_3$ and was equilibrated with 97 % O_2-3 % CO_2 (pH 7.4).

Experimental set-up

A differential high impedance amplifier (built by us according to the version of Thomas) amplified signals from the Ca^{++}-selective microelectrode (V_{CaE}) and a conventional microelectrode filled with 3 M KCl record the membrane potential (V_m). The potential of the bath filled with Tyrode solution was defined as zero for both electrodes. Either simultaneous impalements were made with the conventional and the ion-selective microelectrodes or multiple impalements separately with each electrode. In each case, the impalement sites of both electrodes were less than 200 μm apart from each other. Only those impalements with the Ca^{++}-electrode which showed stable potentials over at least 20 minutes were considered. Usually, the sealing of any leakage around the Ca-electrode was fast (probably because the hydrophobic exterior of the silanized ion-selective electrode

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may improve sealing into the cell membrane [7]). Nevertheless, penetration damage may occur sometimes which would lead to a rise in intracellular Ca^{++} thus, only the lowest values of $(Ca^{++})_i$ measured in an experiment were taken into account. When multiple impalements were made these lowest values were reproducible. Tension, membrane potential and the signal from the Ca^{++}-selective microelectrode were registered on a pen recorder. V_m and V_{CaE} were also displayed on two digital voltmeters and the values of stable impalements were read from these. The subtraction signal of the membrane potential and the Ca^{++}-electrode signal gave V_{diff}. V_{diff} is a measure of the intracellular free $(Ca^{++})_i$ (see Figure 1).

Microelectrodes

The *conventional microelectrodes* were made from glass capillaries with an outer diameter of 1.5 mm. *Ca^{++}-selective microelectrodes* were pulled from 1.5 mm outer diameter Pyrex capillary tubing without filament to a tip-diameter of 1 μm or less. It is known that the hydrophobic Ca^{++}-selective liquid may occasionally back up or escape from the tip of the silanized microelectrode [8]. Comparing tubing with and without filament we noticed that the Ca^{++}-selective sensor is less likely to be displaced in micropipettes made of tubing without filament. Thus, we used this latter type of capillaries for the construction of ion-selective microelectrodes. The displacement of the Ca^{++}-selective liquid is facilitated perhaps due to a mild capillary action of the filament even in the silanized microelectrode. The mechanical stability of the Ca^{++}-selective liquid in the microelectrode was further increased by addition of polyvinylchloride (PVC) to the sensor [9].

Dimethyldichlorsilane was used to make the micropipettes hydrophobic before filling them with the Ca^{++}-sensor. The pipettes were silanized in an oven, as described by Tsien and Rink [10]. The silanized pipettes were filled up to their shank with 0.1 μmol/l $CaCl_2$ solution using a fine Hamilton needle. Adequate pressure was applied from a N_2-gas cylinder to push the solution down to the tip. To apply pressure we placed a pipette in a high pressure tubing tightly fitted in an adjustable socket. This fitting allows gas pressure of up to 13 atm to be easily applied to the micropipette. The high pressure which may be applied using this method also allowed quick filling of very fine-tipped micropipettes (tips of 0.2 μm outer diameter as judged by electron microscopy). Finally the Ca^{++}-selective liquid was pushed into the tip of the micropipettes. With this method, manufacture of Ca^{++}-selective microelectrodes is quickly accomplished. Complete filling of the silanized micropipette with both reference solution and sensor takes about 7–10 minutes.

The Ca^{++}-selective liquid was composed of 10 % (w/w) neutral Ca^{++}-ligand ETH 123 and 1 % sodium tetraphenylborate dissolved in (o-nitrophenyl) octylether. 14 % (w/w) polyvinylchloride (PVC) was added to 86 % original Ca^{++}-selective liquid and dissolved in 3 times the amount of tetrahydrofuran [11]. In microelectrodes with tip-diameters below 1 μm, a Ca^{++}-selective sensor containing PVC is known to give a larger electrode response between 10^{-6} and 10^{-7} M free Ca^{++} than a sensor without PVC [8].

Shortly after their fabrication a potential drift of the Ca^{++}-electrodes in calibration solutions was seen which was largest in the first few hours. The potential changes of most electrodes decreased considerably 4–5 hours after construction [12]. Storing the electrodes overnight was found to be the safest approach in order to defeat the drift [13]. The electrodes then usually showed stable potentials in the calibration

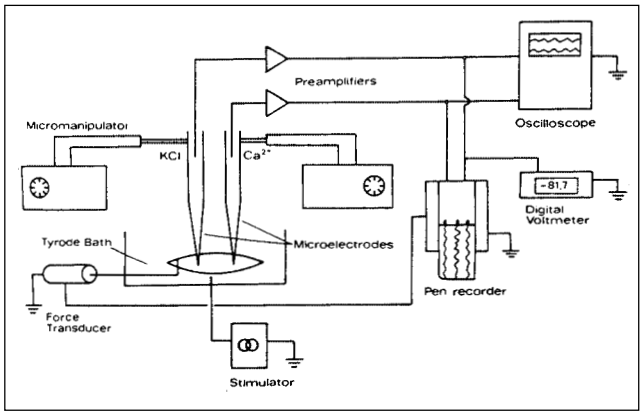

Figure 1. Experimental assembly used for the measurement of $(Ca^{++})_i$ with C^{++}-ionselective microelectrodes

solutions over many hours. Microelectrodes were calibrated before and after each experiment. As experiments were conducted with such electrodes, potential drift was almost never seen during the course of experiments.

The potential of the Ca^{++}-selective microelectrode in given fluids is calibrated against those solutions' free Ca^{++}-concentration. Calibration solutions can be prepared using calcium buffers (for instance EGTA). There has been lack of agreement among authors as to which apparent association constant for the Ca^{++}-buffer complex should be applied under the given circumstances [11, 12]. We therefore did not use calcium buffers. For our calibration solutions we made use of highly purified quartz-distilled, deionized water with very low Ca^{++}-content (always below 10^{-7} mmol/l free Ca^{++}), which we measured by atomic absorption spectroscopy. By simply adding a very small volume ($\sim$ 30 μl) with the appropriate quantity of $CaCl_2$ and the other ions (highly purified) to this water we obtained solutions with 10^{-5}, 10^{-6} and to 10^{-7} M free $(Ca^{++})_i$ (pCa 5 to pCa 7). Dilution of 10^{-2} M $CaCl_2$ solution gave pCa 3 and pCa 4. The free Ca^{++} of all calibration solutions was measured then by atomic absorption spectroscopy and flame photometry and verified at regular intervals. If atomic absorption spectroscopy and highly purified water are available this approach enables preparation of low Ca^{++}-content solutions and gives a reliable proof of the free Ca^{++} of a fluid, thus constituting an alternative to the calculation of free Ca^{++} using association constants. After this procedure KCl 140 mmol/l [14], NaCl 10 mM and $MgCl_2$ 3 mmol/l were added to each of the solutions according to the values of intracellular cation concentrations reported in the literature in order to mimic the cytoplasmic cation composition. Imidazole was used as a pH buffer and the solutions were adjusted to a pH of 7.2.

Statistics

In all groups n = 7 and the standard error of mean (± SEM) is quoted.

Results

Resting tension, total and free intracellular resting calcium in untreated controls

The total calcium determined in the ventricular tissue of the control subjects (tissue excised from the apex) amounted to 4.9 ± 0.8 (n = 7) μmol/g dry weight. The assessment of total Ca content was carried out with AAS. In the same animals right ventricular *free* $(Ca^{++})_i$ (papillary muscle) at rest was 2.0 ± 0.2 x 10^{-7} μmol/l (n = 7) (Table 1, Figure 2). In the same

preparation a mean membrane potential of -87 ± 2 mV was found. The left hand panel in Figure 3 displays original traces of V_m and V_{CaE} in one control experiment. The $(Ca^{++})_i$-concentration is displayed as a dashed line, replacing the original subtraction potential of $V_{CaE} - V_m$ (= V_{diff}). In this experiment pCa was 6.7 (~2×10^{-7} µmol/l). Resting tension at optimal length was ~1 mN. Mean values of the quotient of resting tension and papillary muscle wet weight can be read from Figure 4, which also gives the mean values of animal weight.

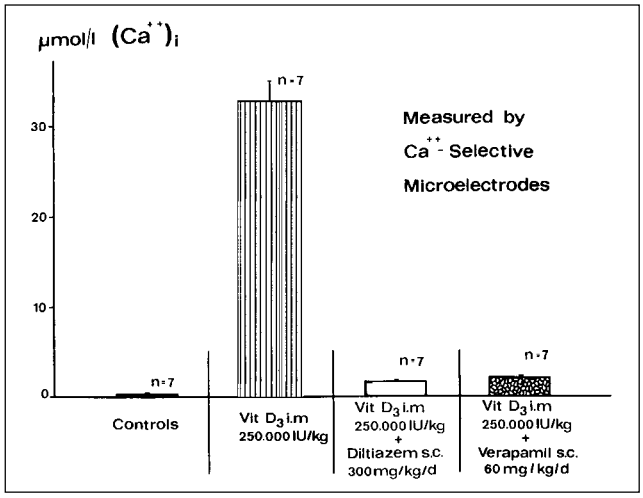

Figure 2. Myocardial $(Ca^{++})_i$ in rat papillary muscle at rest. In those rats which received high dose vit. D_3, $(Ca^{++})_i$ was increased over 160 times. This rather dramatic rise in $(Ca^{++})_i$ could be largely prevented by s.c. administration of diltiazem and verapamil.

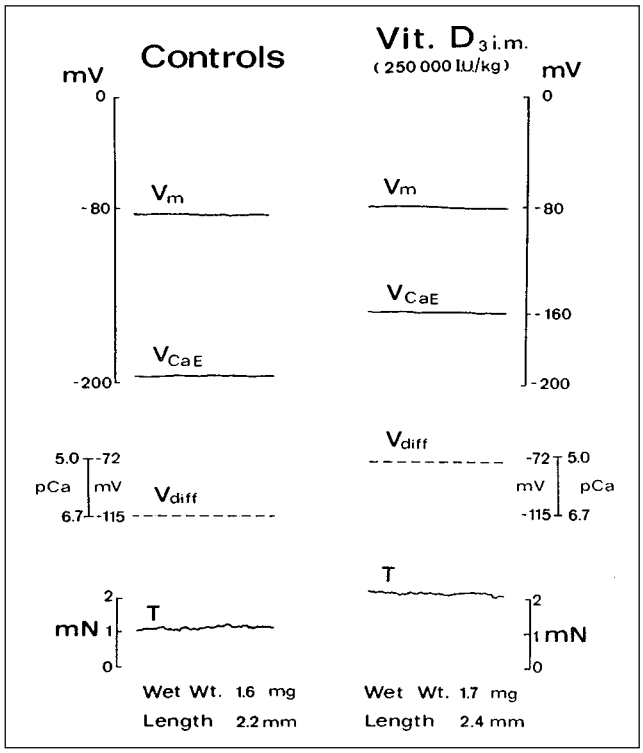

Figure 3. Typical membrane potential (V_m), Ca^{++}-electrode potential (V_{CaE}) and differential potential (V_{diff}) as well as resting tension in control conditions (left hand panel) and in vit. D_3-intoxicated rats (right hand panel). Recordings from two individual experiments.

Resting tension, myocardial total and free calcium are increased in vit. D_3-treated rats

Those rats, however, which were given 250,000 IU of vit. D_3/kg (Vi-De 3 Hydrosol, Wander AG, Bern) showed a massive increase in total tissue Ca amounting to 91.3 ± 38.5 µmol/g dry weight (n = 7), thus displaying some 18 times more total calcium content than the controls. These values are in agreement with earlier findings of our group [4–6]. Conversely, *free* intracellular Ca^{++} was elevated by a factor of more than 160 in comparison to controls showing values of $3.2 \pm 1.0 \times 10^{-5}$ mol/l (n = 7) (Figure 2). The mean membrane potential in these papillary muscles was slightly more depolarized than in the controls (-82 ± 1 mV). Furthermore, in this group the mean resting tension was 2.6 times higher than in the controls (expressed again as the quotient of resting tension per wet weight), usually 86 ± 23 mN/mg (Table 1, Figure 4).

Prevention by diltiazem of vit. D_3-induced myocardial Ca-overload

In those rats which received vit. D_3 (250,000 IU/kg) plus diltiazem (2 x 150 mg s.c./kg/d for 3 days) both total and free myocardial calcium were, although still elevated, significantly less than in the vit. D_3 group. Total calcium was 7.23 ± 1.22 µmol/g (n = 7) in the vit. D_3/diltiazem group (compared to 91 µmol in the vit. D_3 group). Free intracellular calcium in this group was some 20 times less than in the group treated with vit. D_3 alone, amounting to $1.6 \pm 0.8 \times 10^{-6}$ mmol/l. The quotient of resting tension in this group was 36 ± 9 mN/mg, thus smaller than in the vit. D_3 group (Figure 2).

Discussion

Intracellular free resting calcium Ca^{++} in rat myocardium

In our earlier work rats have been used in order to investigate tissue protective effects of Ca-antagonists (eg, high doses of vit. D_3 and/or nicotine, experimental alloxan-induced diabetes mellitus etc., Lit.). Hence, our attention has initially been focused on the resting values of $(Ca^{++})_i$ in the myocardium of untreated rats. In the rat $(Ca^{++})_i$ has not been measured before with ion-selective microelectrodes (Table 1). Papillary muscles were chosen for this purpose because of their geometry which makes them also suitable for *force* measurements. There have been no reports so far on $(Ca^{++})_i$ determined

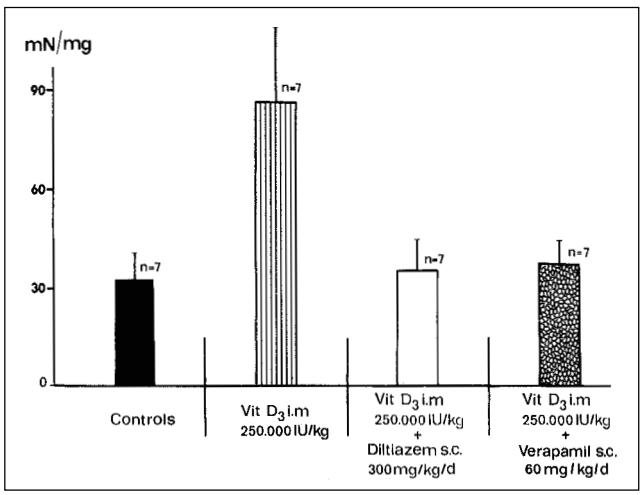

Figure 4. Resting tension increases more than twofold in papillary muscels of vit. D_3-intoxicated rats. The administration of diltiazem or verapamil attenuates this increase in resting tension.

with Ca^{++}-selective microelectrodes measured in *rat* heart muscle. There have been, however, scarce reports on $(Ca^{++})_i$ measured by ion-selective microelectrodes in heart muscle of other species (eg, in sheep myocardium $(Ca)_i$ was 3.6×10^{-7} mol/l [9], 2.6×10^{-7} mol/l in ferret [10], 2.7×10^{-7} in rabbit [14] and 2.9×10^{-7} in guinea pig heart [15]. $(Ca^{++})_i$ in *rat* ventricular myocardium has, however, also been assessed using other techniques: for example, measurements with the photoprotein aequorin and the fluorescent indicator quin 2 [24] gave similar readings. Advantages, pitfalls and the validity of the method (Ca^{++}-selective microelectrodes) have been discussed in an earlier paper [16]. Briefly, these microelectrodes are particularly well suited for determining Ca^{++} ion activity (which equals the concentration of ions available for interaction with the contractile apparatus). These electrodes allow the measurement *without* previous tissue disaggregation. Furthermore, this technique measures the membrane potential, and thus provides important information about the electrode state of the preparation [17].

The intracellular calcium concentration of the myocardium plays a key role in changes of inotropy and genesis of cell necrosis. The idea of this study was to perform comparative measurements of total calcium content of tissue and corresponding intracellular free calcium. We were able to show that vit. D_3-induced Ca^{++}-overload of the myocardium increases the *total* calcium content of the tissue [4, 5] as well as the intracellular *free* calcium concentration. This rise in free intracellular Ca^{++} is also reflected in a marked increase in resting tension. On the other hand we demonstrated that diltiazem prevents this rise in $(Ca^{++})_i$ in rats treated with a high-dose of vit. D_3. The mechanism of this cardio-protective effect of diltiazem is unclear. It is difficult to imagine that this prevention of $(Ca^{++})_i$-overload by diltiazem is based on Ca^{++}-channels, since vit. D_3 is not known to act via these channels. Thus, one must consider a further, so far unknown, action of specific Ca^{++}antagonists upon Ca^{++}-transport through cell membranes.

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This manuscript is dedicated to the memory of my teacher in electrophysiology Prof. Dr. med. Dr. h.c. mult. Albrecht Fleckenstein. It was the last scientific work we were allowed to perform together.

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