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Origin of concurrent ATPase activities in skinned cardiac trabeculae from rat

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1. To determine the rate of ATP turnover by the sarcoplasmic reticulum (SR) Ca^{2+} pump in cardiac muscle, and to assess the contributions of other ATPase activities to the overall ATP turnover rate, ATPase activity and isometric force production were studied in saponin-skinned trabeculae from rat. ATP hydrolysis was enzymatically coupled to the oxidation of NADH; the concentration of NADH was monitored photometrically. All measurements were performed at 20 ± 1 °C and pH 7·0. Resting sarcomere length was adjusted to 2·1 μm. All solutions contained 5 mM caffeine to ensure continuous release of Ca^{2+} from the SR.

2. The Ca^{2+}-independent ATPase activity, determined in relaxing solution (pCa 9), amounted to 130 ± 13 μM s^{-1} (mean ± S.E.M., $n = 7$) at the beginning of an experiment. During subsequent measurements in relaxing solution, a decrease in ATPase activity was observed, indicative of loss of membrane-bound ATPase activity. The steady-state Ca^{2+}-independent (basal) ATPase activity was 83 ± 5 μM s^{-1} ($n = 66$).

3. Treatment of saponin-skinned preparations with Triton X-100 abolished 50 μM s^{-1} (60%) of the basal ATPase activity. Addition of ouabain (1 mM) suppressed 14 ± 5% of the basal activity, whereas 8 ± 3% was suppressed by 20 μM cyclopiazonic acid (CPA). It is argued that 31 μM s^{-1} of the basal ATPase activity may be associated with MgATPase from the transverse tubular system.

4. The maximal Ca^{2+}-activated ATPase activity, i.e. the total ATPase activity (determined in activating solution, pCa 4·3) corrected for basal ATPase activity, was found to be 409 ± 15 μM s^{-1} ($n = 66$). Experiments with CPA indicated that at least 9 ± 6% of the maximal Ca^{2+}-activated ATPase activity originates from the sarcoplasmic Ca^{2+} pump. These experiments indicate that the rate of ATP consumption by the SR Ca^{2+} transporting ATPase amounts to at least 37 μM s^{-1}.

5. Treatment of preparations with Triton X-100 abolished 15 ± 3% of the maximal Ca^{2+}-activated ATPase activity, indicating that 15 ± 3% of the maximal Ca^{2+}-activated ATPase activity is membrane bound.

6. Variation of free [Ca^{2+}] indicated that apart from the actomyosin ATPase activity a second Ca^{2+}-dependent ATPase activity contributed to the overall ATP turnover rate. This activity was half-maximal at pCa 6·21, and probably reflects the SR Ca^{2+} transporting ATPase. It constituted 18 ± 3% of the Ca^{2+}-dependent ATPase activity, yielding an upper limit for the SR Ca^{2+} transporting ATPase activity of 74 μM s^{-1}.

The major energy-requiring processes in cardiac muscle are activation, involving the release and uptake of Ca^{2+} by the sarcoplasmic reticulum (SR), and force production by the myofibrils. Energy for these processes is provided by the hydrolysis of adenosine 5′-triphosphate (ATP). The properties of the cardiac SR have been extensively studied in isolated SR fragments and vesicles. Moreover, experiments in both intact and permeabilized cardiac myocytes have given insights into the processes involved in the regulation of the flow of Ca^{2+} inside the cell (for review see, e.g. Inesi, 1985; Barry & Bridge, 1993).

However, less is known about the energy cost of these Ca^{2+} transporting processes. To determine the energetic aspects of activation and Ca^{2+} handling, several authors have measured the rate of heat production in intact trabeculae

and papillary muscles (e.g. Gibbs, 1978; Loiselle & Gibbs, 1979; Daut & Elzinga, 1988). However, it is difficult to discriminate between the different origins of heat production by various processes in intact preparations. Hence, there still exists considerable uncertainty regarding the maximal activity and [Ca^{2+}] dependence of the various ATPases involved in resting metabolism and in the working heart. We therefore studied the ATP turnover rate in saponin-skinned cardiac trabeculae. Saponin permeabilizes the sarcolemma of myocytes in trabeculae, whereas the SR and the contractile apparatus are left functionally intact (Endo & Iino, 1980; Kurebayashi & Ogawa, 1991). Using saponin-skinned preparations, it is possible to control the composition of the intracellular environment. This is particularly advantageous when studying the SR, since its Ca^{2+}-dependent ATPase activity can be studied by varying the intracellular Ca^{2+} concentration. Also, the effects of inhibitors of ion pumps such as the SR Ca^{2+} pump and the Na^+–K^+ pump on the overall ATPase activity can be readily observed in skinned preparations.

In the experiments described in this paper, we investigated the relative contribution of the SR ATPase activity to the overall ATP turnover. To discriminate between SR ATPase activity and other activities, such as actomyosin ATPase activity, we used cyclopiazonic acid (CPA) and thapsigargin, which have been reported to be specific inhibitors of the SR Ca^{2+} pump (Seidler, Jona, Vegh & Martonosi, 1989; Kijima, Ogunbunmi & Fleisher, 1991).

Apart from the Ca^{2+} transporting ATPase activity, there are several other membrane-bound ion pumps active in cardiac muscle, e.g. the Na^+–K^+ transporter and the Na^+–Ca^{2+} exchanger. Furthermore, a Ca^{2+}-independent MgATPase in vesicles from the T-tubules system (from skeletal and cardiac muscle) has been reported (Beeler, Gable & Keffer, 1983; Beeler, Wang, Gable & Lee, 1985). In order to obtain insights into the relative contributions of these membrane-bound ATPase activities to the overall ATP turnover rate, experiments with Triton X-100 and ouabain were also performed.

In this study, we found that the maximal SR ATPase activity is considerably smaller than the values found in skeletal muscle. Furthermore, the contribution to the overall ATPase activity from other, Ca^{2+}-independent ATPases was found to be substantial.

METHODS

Preparation

Adult male rats (~350 g body weight, Wistar and LBN-F1 strains) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (6 mg (100 g)$^{-1}$), and the heart was rapidly excised. The heart was connected to a Langendorff perfusion system and perfused with oxygenated Tyrode solution consisting of (mM): NaCl, 128; KCl, 5; $CaCl_2$, 1·4; $MgCl_2$, 1; $NaHCO_3$, 20; Na_2HPO_4, 0·4; and glucose, 11; equilibrated at pH 7·4 with 95% O_2–5% CO_2. When the vasculature was visibly clear of blood, the Tyrode solution was changed for one containing 20 mM 2,3-butanedione monoxime (BDM; Sigma), which effectively inhibits contracture and muscle damage during dissection (Mulieri, Hasenfuss, Ittleman, Blanchard & Alpert, 1989). When the heart had stopped beating, thin unbranched trabeculae were dissected carefully from the right ventricle. The preparations had a diameter between 70 and 250 μm (140 ± 3 μm, mean ± S.E.M., $n = 66$) and a length between 1·35 and 3·10 mm (1·85 ± 0·05 mm). Immediately after dissection, the preparations were transferred to a dish containing cold relaxing solution (pCa 9) and 50 μg ml^{-1} saponin (Merck), and kept on ice for 30–45 min. This relaxing solution used phosphocreatine (PCr) as an ATP-regenerating system, and had the following composition (mM): Na_2ATP, 6·0; $MgCl_2$, 6·6; EGTA, 20; PCr (added as Na_2PCr), 10; *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (Bes), 100. Ionic strength was adjusted to 200 mM using potassium propionate ($KCH_2CH_3CH_3$), and pH was adjusted to 7·1 by addition of KOH. The skinned preparations were mounted in the experimental set-up using aluminum T-clips, as described previously by Stienen, Roosemalen, Wilson & Elzinga (1990).

Apparatus

Isometric force was measured using a force transducer element (AE801, SensoNor, Horten, Norway). Sarcomere length was determined in relaxing solution by means of He–Ne laser diffraction (model 1125; Uniphase, San Jose, CA, USA). The preparation could be manually transferred between baths with a volume of 80 μl containing relaxing, pre-activating and activating solutions (solution composition listed below). Measurements were carried out in the measurement chamber, which had a volume of about 30 μl. The solution in this chamber was continuously stirred by motor-driven vibration of a diaphragm at the bottom of the chamber (frequency about 3 Hz). ATPase activity was measured using an enzyme-linked assay described in detail previously (Glyn & Sleep, 1985; Ebus, Stienen & Elzinga, 1994). In short, ADP was resynthesized to ATP, and this reaction was coupled to the oxidation of NADH to NAD^+, catalysed by pyruvate kinase (PK) and lactic dehydrogenase (LDH), respectively. The breakdown of NADH was determined photometrically from the absorbance by NADH of near-UV light (340 nm) that passed beneath the preparation through the measuring chamber. The absorbance signal was found to be linearly related to the NADH concentration in the range between 0·2 and 2 mM. During a measurement, the NADH concentration decreased typically from 0·8 to 0·7 mM. After a measurement, calibration of the absorbance signal was carried out by injecting several aliquots of 0·05 μl of 10 mM ADP (i.e. 0·5 nmol) into the measuring chamber. The absorbance and force signals were filtered at 2·5 Hz (−12 dB oct^{-1}), sampled at 5 Hz and recorded on an Olivetti M280 personal computer.

Experimental protocol

Measurements were performed in solutions containing phosphoenolpyruvate (PEP) as an ATP-regenerating system (solution composition listed below). Measurements were done in relaxing solution (pCa 9), activating solution (pCa 4·3) and solutions of intermediate pCa. Solutions of intermediate pCa were prepared by mixing appropriate amounts of relaxing and activating solution. When a measurement in relaxing solution was performed, preparations were incubated in the 80 μl bath containing relaxing solution for at least 4 min and then transferred directly to the measuring chamber. When a measurement in activating solution or a solution with intermediate pCa was performed, preparations were incubated in relaxing solution for at least 4 min, then transferred to pre-activating

solution and incubated for at least 3 min. Then the preparation was transferred to the measuring chamber. After a measuring period of about 60 s, the preparation was transferred back to the bath containing relaxing solution. Solutions were refreshed regularly, to ensure that evaporation of the solutions and ATP consumption by the preparation did not significantly change their composition.

The composition of the solutions was calculated using the equilibrium constants given by Fabiato & Fabiato (1979), corrected by 0·12 log units as indicated in Fabiato (1981). The equilibrium constants for PEP were given by Vianna (1975). Relaxing solution contained (mM): Na_2ATP, 5·86; $MgCl_2$, 7·76; and EGTA, 20. Pre-activating solution contained: Na_2ATP, 5·86; $MgCl_2$, 7·35; EGTA, 0·5; and hexamethylenediamine *N,N,N′,N′*-tetraacetic acid (HDTA), 19·5. Activating solution contained (mM): Na_2ATP, 5·97; $MgCl_2$, 7·24; and CaEGTA, 20 (pCa 4·3). CaEGTA was made by mixing equimolar amounts of $CaCO_3$ and EGTA. All solutions contained 100 mM Bes, 10 mM PEP (added as potassium salt), 0·8 mM NADH, 4 mg ml^{-1} pyruvate kinase (500 U (mg protein)$^{-1}$; Sigma, P-9136) and 0·24 mg ml^{-1} lactic dehydrogenase (800 U (mg protein)$^{-1}$; Sigma, L-1254). In addition, all solutions contained 5 mM caffeine to ensure continuous stimulation of the Ca^{2+} release channels of the SR. Oligomycin-B (10 μM) and sodium azide (5 mM) were added to inhibit ATP production and/or consumption by the mitochondria. These solutions also contained the myokinase inhibitor p^1,p^5-di(adenosine-5′) pentaphosphate (0·2 mM; Boehringer–Mannheim). The free Mg^{2+} and MgATP concentrations were 1 and 5 mM, respectively. Ionic strength was adjusted to 200 mM by adding potassium propionate, and pH was adjusted to 7·0 by addition of KOH.

Control measurements were carried out at saturating calcium concentration (pCa 4·3). It was ensured that the [Ca^{2+}] was saturating by adding extra Ca^{2+} from a concentrated Ca^{2+} stock during maximal Ca^{2+} activation. After the first measurement (at pCa 4·3), sarcomere length, determined in relaxing solution, was readjusted if necessary, and the length, width and depth of the preparation were measured at ×50 magnification. In general, resting sarcomere length remained stable after this adjustment. A second measurement at pCa 4·3 provided the control value for isometric force and ATPase activity. After two measurements, another control measurement was carried out. Force and ATPase activity were corrected for fibre deterioration by linear interpolation between control values. The intermediate results were normalized to the interpolated control values. When isometric force was less than 80% of the initial control value, the measurements after the preceding control were discarded. In some experiments, preparations underwent treatment with the detergent Triton X-100 (1% v/v) for at least 2 h at room temperature (20 ± 1 °C). In these experiments, preparations that showed a variation in force over 10% before and after Triton treatment were discarded. Preparations with both width and depth larger than 160 μm were not used in these experiments.

ATPase activity was determined from linear regression of the absorbance signal. To correct for artifacts such as NADH bleaching under the intense UV light, contaminant ATPase activities in LDH and evaporation of the solution in the measuring chamber, the slope of the absorbance signal was recorded after the fibre had been transferred from the measuring chamber to the bath containing relaxing solution. This slope was then subtracted from the absorbance signal measured when the preparation was in the measuring chamber.

Because surface tension in the measuring chamber influenced the baseline of the force transducer in the measuring chamber, passive tension was determined during a measurement in relaxing solution (pCa 9). This value was subtracted afterwards from the values that were found in solutions with pCa lower than 9.

In fibres which underwent additional Triton X-100 treatment a decrease of about 10% in fibre diameter was observed, possibly as a result of mitochondrial damage and/or dissolution. For the ease of comparison, all values are expressed with respect to the fibre dimensions that were determined in saponin-skinned preparations prior to Triton treatment.

Statistics

Curve fitting was performed using curve-fitting software (Abelbeck Software, Reading, PA, USA) based on the Marquardt algorithm. The Ca^{2+} dependence of ATPase activity was investigated by fitting the data with a Hill curve. This curve describes the dependence of ATPase activity on Ca^{2+} as:

$$\text{ATPase activity} = A_{max}\frac{[Ca^{2+}]^{n_H}}{[Ca^{2+}]_{50}^{n_H} + [Ca^{2+}]^{n_H}},$$

where A_{max} is the ATPase activity at saturating Ca^{2+} concentration (pCa 4·3), $[Ca^{2+}]_{50}$ is the Ca^{2+} concentration at half-maximal activity, and n_H is the coefficient of steepness (Hill coefficient). Data were also fitted as a sum of two Hill curves.

Data values are given as means ± S.E.M. of *n* experiments. Student's *t* test was used to test significance of differences at a 0·05 level of significance ($P < 0·05$).

Chemicals

Cyclopiazonic acid (CPA) and Triton X-100 were supplied by Sigma. 2,5-di(*tert*-butyl)hydroquinone (TBQ) and thapsigargin (TG) were supplied by Alamone Labs (Jerusalem).

RESULTS

In Fig. 1, the absorbance signals, from which the rate of ATP consumption is derived, and isometric force are shown from measurements at pCa 9 and pCa 4·3.

Figure 1*A* shows the absorbance signal and isometric force during a measurement in relaxing solution (pCa 9). The recording started while the preparation was immersed in the 80 μl bath containing relaxing solution. After the preparation was transferred to the measuring chamber, the absorbance started to decrease. Small differences in NADH concentration between the solution in the measuring chamber and the solution carried with the preparation caused a transient change in the absorbance signal. After a short equilibration period, the absorbance signal and therefore the NADH concentration in the measuring chamber decreased linearly with time. After a sufficiently long measuring period, the preparation was transferred back to the bath containing relaxing solution. Thereafter, the absorbance baseline was measured to correct for small changes in the NADH concentration due to bleaching of NADH, evaporation and a small amount of contaminant ATPase activity in the enzymes used. Finally, 0·5 nmol ADP was injected into the measuring chamber to calibrate the absorbance signal.

Basal ATPase activity

During every experiment, a control measurement in relaxing solution was included, to determine the contribution of Ca^{2+}-independent ATPase activity to the overall ATP turnover rate. Repeated measurements in relaxing solution in saponin-skinned preparations revealed that the level of Ca^{2+}-independent ATPase activity was not constant. After adjustment of resting sarcomere length to 2·1 μm, repeated measurements were performed in relaxing solution. The results of these experiments are shown in Fig. 2. These experiments show that the Ca^{2+}-independent ATPase activity decreased monotonically during consecutive measurements from 130 μM s^{-1} (measurement near 1) to a steady level of 83 ± 3 μM s^{-1} ($n = 66$).

In all experiments, a number of measurements in relaxing solution were performed until the Ca^{2+}-independent ATPase activity reached a stable level. Typically, ATPase activity stabilized after two to three measurements. Then, further measurements were performed. At the end of a series of measurements (typically 5–10 measurements in solutions with various Ca^{2+} concentrations), the basal ATPase activity was measured again. It was found that once a steady level had been reached during the initial repeated measurements in relaxing solution, basal ATPase activity remained stable throughout an experiment. This decrease in Ca^{2+}-independent ATPase activity was also observed when the initial measurements were performed in activating instead of relaxing solution.

To determine whether the mitochondria in the preparation contributed to the decrease in NADH absorbance by oxidizing exogenous NADH, we measured the absorbance when the preparations were immersed in solutions which

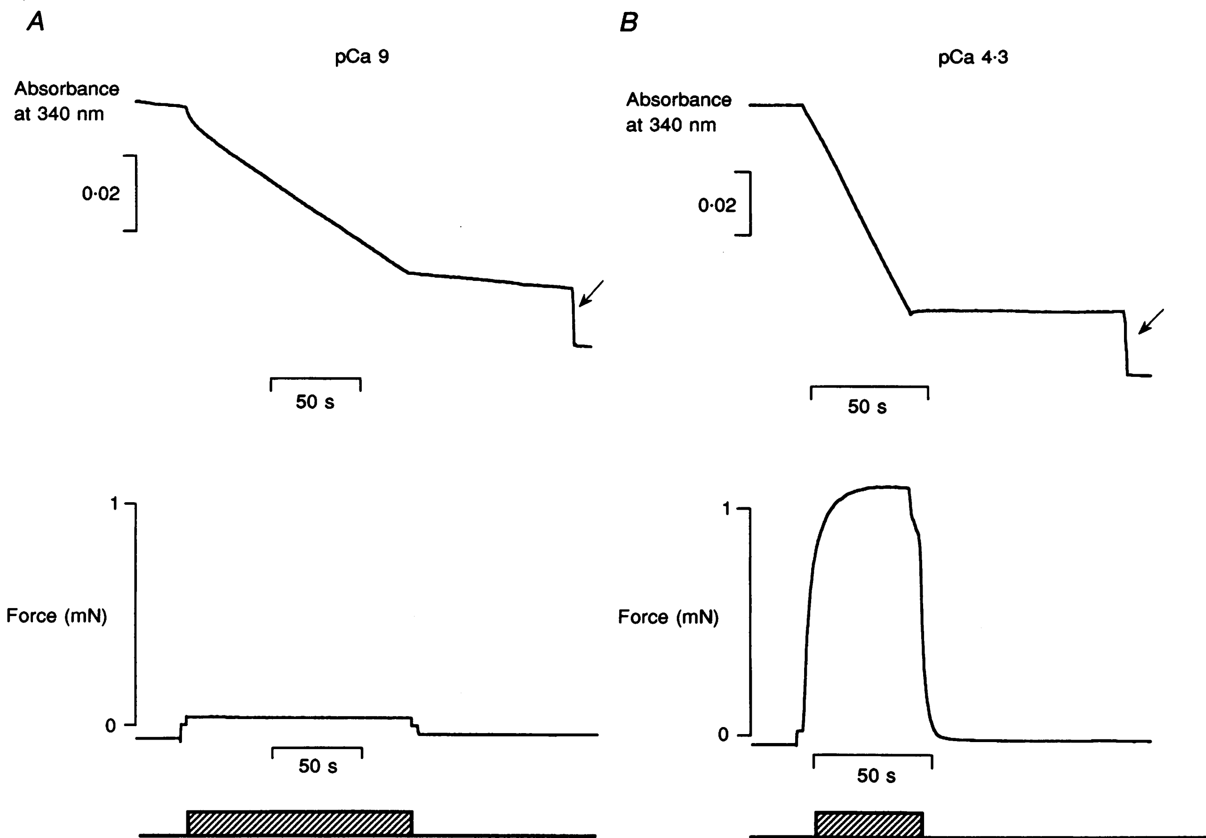

Figure 1. NADH absorbance and force production, measured in relaxing and activating solution

A, absorbance of NADH in the measuring chamber (upper panel) and force (lower panel) during a measurement in relaxing solution (pCa 9). The hatched bar marks the time the preparation was in the measuring chamber. After transferring the preparation to the measuring chamber, the absorbance started to decrease, indicating ATP turnover. After the measurement, the preparation was replaced in the 80 μl bath, which also contained relaxing solution. After measuring the absorbance without the preparation present, 0·5 nmol ADP, marked by the arrow, was injected into the measuring chamber to calibrate the absorbance signal. The small staircase changes in force upon insertion of the preparation in the measuring chamber are due to differences in the force transducer baseline in various chambers. *B*, absorbance and isometric force during maximal Ca^{2+} activation. When the fibre was transferred from the bath containing pre-activating solution to the measuring chamber containing activating solution (pCa 4·3), active force developed and absorbance started to decrease. After about 50 s, the preparation was placed back in relaxing solution, and force quickly fell to the resting level.

used phosphocreatine (PCr, 10 mM) as an ATP regenerating system to which NADH (0·8 mM) was added. The absorbance did not change significantly during measurements in activating (0·6 ± 0·9% of total ATP turnover rate; $n = 3$) and relaxing solution (1·4 ± 0·2% of total ATP turnover rate; $n = 4$).

During the actual measurements of ATPase activity, preparations typically spent about 10 min in PEP-based relaxing solution containing azide and oligomycin prior to a measurement. During some experiments, the preincubation period in relaxing solution was 30 min or more, but this did not seem to influence the extent nor the time course of the decrease in Ca^{2+}-independent ATPase activity. The decline of activity during subsequent measurements predominantly depended upon the number of measurements, rather than upon the total time spent in relaxing and pre-activating solution. It was also observed that thicker preparations required more repeated measurements before a steady level of Ca^{2+}-independent ATPase activity was reached. These observations suggest that the decrease in ATPase activity is mainly caused by washout of membrane fractions with bound ATPases which occurs while the preparations are immersed in the measuring chamber, in which the solution is thoroughly stirred. The time that preparations spent in the chamber was about the same during all measurements, and relatively short in comparison with the time that preparations spent in the non-stirred 80 μl baths. This indicates that the decrease of ATPase activity was drastically accelerated by stirring. During several measurements in thick preparations, it was noted that the baseline of the absorbance signal after the incubation was steeper (and therefore absorbance decreased faster) than prior to the incubation in the measuring chamber. This supports the notion that the decrease in ATPase activity is caused by washing ATPase activity out of the preparation. This ATPase activity remains in the solution in the measuring chamber after the preparation is placed back in relaxing solution.

All absorbance signals were corrected for the change in absorbance that was measured after the preparation had been removed from the measuring chamber. However, during the initial measurements, washed out ATPase activity remained in the measuring chamber and therefore caused an additional decrease in absorbance. This additional decline was not taken into account, since the total absorbance after removal of the preparation from the measuring chamber served as a baseline. Thus, the ATP turnover rate determined from the change in absorbance after correction for this baseline represented the ATPase activity that remained in the preparation after the measurement. To obtain an estimate of the Ca^{2+}-independent ATPase activity present before the first

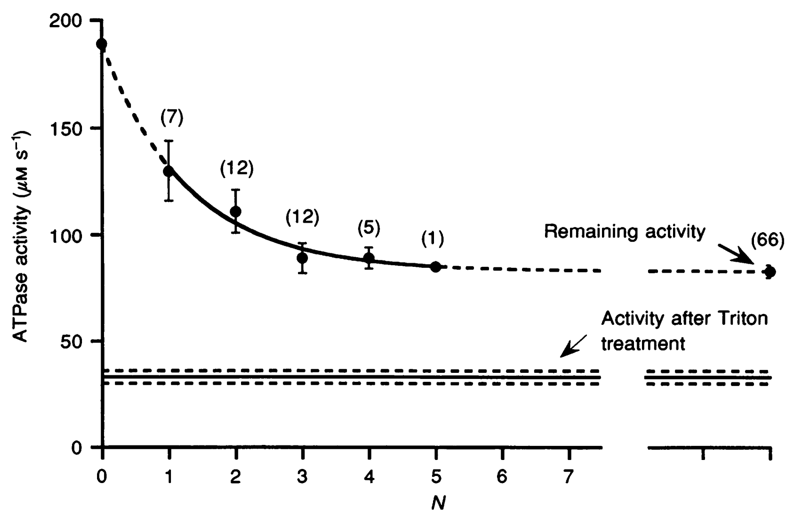

Figure 2. Washout of Ca^{2+}-independent ATPase activity

Data points denote Ca^{2+}-independent ATPase activity, determined in relaxing solution, during consecutive measurements. The numbers on the X-axis represent the number of experiments done at the Nth measurement in relaxing solution. The number of data points at $N = 1$ is less than the number of points at $N = 2$, because some experiments started with a measurement in activating instead of relaxing solution. The data were fitted using a mono-exponential curve, which yielded:

$$\text{ATPase activity} = 83{\cdot}0 + 105{\cdot}9e^{-0{\cdot}77N}\ \mu\text{M s}^{-1},$$

where N represents the Nth consecutive measurement during an experiment. Extrapolation of this fit to the point $N = 0$ is represented by the dashed part of the curve to the left. The dashed part of the curve to the right and the final data point represent the remaining activity that was not washed out. The lower line in this figure represents the basal ATPase activity measured in preparations which had been treated with Triton X-100.

measurement, the exponential curve fitted in Fig. 2 was extrapolated to $N = 0$. This yielded a maximum value of 189 ± 54 μM s^{-1} for the initial basal ATPase activity.

To determine whether the remaining basal ATPase activity in saponin-skinned muscle was membrane bound, preparations were subsequently skinned in 1% (v/v) Triton X-100 for 2 h at room temperature. Since all membrane structures are dissolved by Triton (Miller, Elder & Smith, 1985), membrane-bound ATPase activities were expected to be completely abolished by this treatment. It can be seen in Fig. 2 that Triton treatment indeed abolished a large fraction (60%, or 50 μM s^{-1}) of the basal ATPase activity that remained in saponin-skinned preparations. In Triton-treated preparations, the level of Ca^{2+}-independent ATPase activity did not change during an experiment.

Values for the contribution of the Na^+–K^+ transporter in the sarcolemma to the basal metabolism in intact cardiac muscle have been reported by Schramm, Klieber & Daut (1994). To determine whether the Na^+–K^+ pumps contributed to the basal ATP turnover rate in saponin-skinned muscle, ouabain, a specific inhibitor of this ATPase activity, was added during measurements in relaxing solution. Ouabain was added from a 50 mM stock, dissolved in water. When 1 mM ouabain was added to relaxing solution, basal ATPase activity was reduced to 86 ± 5% ($n = 9$). Ouabain had no effect on passive force in relaxing solution. In five additional experiments, the effects of 0, 1 and 10 mM ouabain in the presence of 5 mM caffeine were measured. In these experiments, ouabain was added as a solid, due to the low solubility of this compound at high concentrations. These paired experiments showed that basal ATPase activity was slightly but significantly depressed to 95 ± 3% in the presence of 1 mM ouabain, and to 86 ± 3% in the presence of 10 mM ouabain. Although 10 mM ouabain depressed basal ATPase activity slightly more than 1 mM, the effects of 1 and 10 mM ouabain were not significantly different (paired t test, $P < 0.05$). These experiments suggest that under our experimental conditions 1 mM ouabain exerts a near-maximal effect on the Na^+–K^+ pump.

Cyclopiazonic acid (CPA) is reported to be a specific inhibitor of the SR Ca^{2+} pump (Seidler *et al.* 1989). We used CPA to determine whether the SR Ca^{2+} pump contributes to the basal ATP turnover rate. To ensure a maximal effect of CPA, preparations were allowed to incubate in CPA for periods of 30 min up to 1 h. In previous experiments, the effects of 10 μM CPA on Triton-skinned preparations were investigated (Ebus *et al.* 1994). These experiments showed no significant effect of CPA or ethanol (0·5%) on force or ATPase activity, which indicates that the contractile apparatus is not affected by CPA. When 20 μM CPA was added to relaxing solution in saponin-skinned preparations, we observed a small decrease in basal ATPase activity to 92 ± 3% of the control value in relaxing solution without CPA. CPA was added from a 2 mM stock dissolved in ethanol, and therefore we also measured the effect of 1% (v/v) ethanol on ATPase activity and force. Ethanol (1%) had no significant effect on passive force or ATPase activity in relaxing solution. To determine whether the influence of CPA was dependent on the presence of caffeine, we tested the effects of 20 μM CPA in solutions without caffeine. In solutions with 20 μM CPA and no caffeine, basal ATPase activity was depressed to 94 ± 2% of the control value without CPA in the presence of 5 mM caffeine. This decrease is not significantly different from the decrease observed when 20 μM CPA was added in the presence of 5 mM caffeine (92 ± 3%). The effects of CPA in relaxing solution therefore did not appear to be dependent on the presence of caffeine *per se.*

To determine the effects of caffeine on the basal ATPase activity, paired experiments were performed in the presence of 0, 5 and 25 mM caffeine ($n = 4$). Both 5 and 25 mM caffeine showed no significant difference from the control value without caffeine (103 ± 1 and 101 ± 1%, respectively).

Maximal Ca^{2+}-activated ATPase activity

Isometric force and absorbance during a maximal Ca^{2+} activation are shown in Fig. 1*B*. When the fibre was transferred to the measuring chamber containing activating solution, isometric force developed and the absorbance quickly decreased. Upon reaching a steady state, the decrease in absorbance and the level of isometric force were measured for about 20 s. Then the preparation was transferred back to relaxing solution, and force quickly fell to the resting level. After measuring the absorbance without the preparation present, 0·5 nmol of ADP was injected to calibrate the absorbance signal. The average isometric force in maximally Ca^{2+}-activated preparations was 39 ± 1 kN m^{-2} ($n = 66$). Maximal Ca^{2+}-activated ATPase activity was determined by subtracting the value for basal ATPase activity (determined in relaxing solution) from the ATP turnover rate that was measured in activating solution. The maximal Ca^{2+}-activated ATPase activity (expressed in micromoles per preparation volume per second) was 409 ± 15 μM s^{-1} ($n = 66$).

To determine whether 5 mM caffeine was sufficient to ensure continuous and maximal release of Ca^{2+} from the SR, experiments were performed in the presence of 0, 5 and 25 mM caffeine. Measurements in activating solution without caffeine showed a small, non-significant increase to 106 ± 7% ($n = 2$) in ATPase activity with respect to the control value with 5 mM caffeine; 25 mM caffeine ($n = 5$) reversibly decreased ATPase activity and force to 81 ± 5% and 76 ± 8%, respectively, of the control value with 5 mM caffeine. Although the decrease in ATPase activity in the presence of 25 mM caffeine was slightly less than the decrease in force, this difference was not significant. Since these relatively high concentrations of caffeine are likely to affect contractile properties, it is not clear whether the smaller decrease in ATPase activity reflects additional

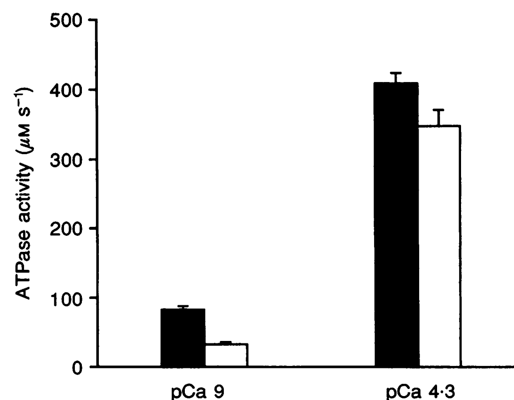

Figure 3. ATPase activity in saponin- and Triton-skinned preparations

The filled bars show basal and maximal Ca^{2+}-activated ATPase activity in saponin-skinned preparations at pCa 9 and 4·3. The open bars show basal and maximal Ca^{2+}-activated ATPase activity in Triton-skinned preparations. ATPase activity is expressed in μM s^{-1} using the dimensions of the saponin-skinned preparations prior to Triton treatment.

stimulation of the SR Ca^{2+} pump. To investigate whether the SR Ca^{2+} ATPase activity was indeed maximally stimulated, we also investigated the effects of other agents that are known to stimulate the SR Ca^{2+} pump.

It has been shown that adding low concentrations of detergents, such as Triton X-100, can have a stimulating effect on the Ca^{2+} ion pump. We therefore studied the effect of 0·1 % (v/v) Triton X-100 on force and ATPase activity in activating solution containing 5 mM caffeine. It was found that Triton had no significant effect on maximal Ca^{2+}-activated ATPase activity (99 ± 11 %, $n = 5$).

In five paired experiments, the effects of the Ca^{2+} ionophore A23187 (20 μM) were measured during maximal Ca^{2+} activation. After measurements in control solutions with and without caffeine, preparations were incubated in solutions containing A23187 for at least 10 min. Addition of 20 μM A23187 to solutions without caffeine had no significant effect on force (98 ± 3 %) or ATPase activity (99 ± 1 %) with respect to the control value without caffeine and A23187. Addition of 20 μM A23187 in the presence of 5 mM caffeine also had no significant effect on force (95 ± 3 %) and ATPase activity (102 ± 4 %).

To determine the contribution of membrane-bound ATPase activities to the maximal Ca^{2+}-activated ATPase activity in saponin-skinned preparations, preparations underwent treatment with Triton for at least 2 h at room temperature. The effects of Triton treatment on the maximal Ca^{2+}-activated ATPase activity (determined in activating solution) are shown in Fig. 3. This figure shows basal and maximal Ca^{2+}-activated ATPase activity in saponin-skinned and Triton-skinned preparations. In this set of experiments, maximal Ca^{2+}-activated ATPase activity

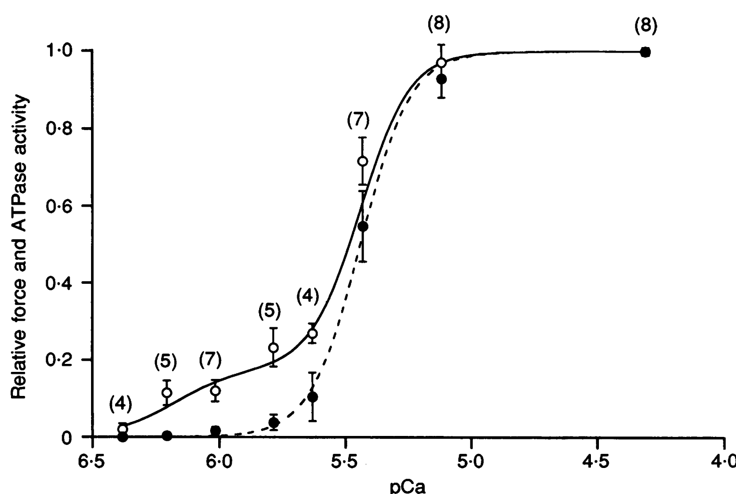

Figure 4. ATPase activity and isometric force in saponin-skinned preparations as a function of the free Ca^{2+} concentration

All values were normalized to the control values at pCa 4·3. ○, ATPase activity; ●, isometric force. The number of experiments is indicated in parentheses at each data point. Data were corrected for basal ATPase activity and passive force before normalization. Isometric force was fitted using a Hill curve (dashed line), yielding a pCa_{50} (pCa for half-maximal activity) of 5·44 ± 0·07 and coefficient of steepness $n_H = 4·4 \pm 0·4$. ATPase activity in saponin-skinned preparations was fitted (continuous line) using the sum of the Hill curve that was determined for isometric force and a second Hill curve, as indicated in the text. The second curve comprised 18 ± 3 % of this sum, with a pCa_{50} of 6·21 ± 0·09 and $n_H = 3·5 \pm 1·6$.

decreased by 15 ± 2% from 404 ± 22 to 348 ± 23 μM s^{-1} after Triton treatment ($n = 20$). This indicates that 15% of the maximal Ca^{2+}-activated ATPase activity in saponin-skinned preparations is membrane bound. We also found that the diameter of preparations decreased on average by 11% after Triton treatment. This is probably due to the dissolution of mitochondria by Triton. For the ease of comparison, the values of isometric force and ATPase activity that were determined after Triton treatment are expressed with respect to the dimensions measured in saponin-skinned muscle. This decrease in fibre diameter should be taken into account in comparing values in this study with our results in Triton-skinned trabeculae reported earlier (Ebus *et al.* 1994).

To investigate further the origin of this Ca^{2+}-dependent ATPase activity, we measured ATPase activity and isometric force at various Ca^{2+} concentrations ($n = 8$). The results of these experiments are shown in Fig. 4. It was found earlier that ATPase activity and force in Triton-skinned preparations are proportional after correction for basal ATPase activity (Stienen, Papp & Elzinga, 1993). Since the contractile apparatus is functionally intact in saponin-skinned preparations, this actomyosin ATPase activity is also present in saponin-skinned preparations. Since the Ca^{2+} dependence of the actomyosin ATPase activity in Triton-skinned preparations can be well fitted by a Hill curve, the ATPase activity in saponin-skinned preparations was fitted by two Hill curves, one representing actomyosin activity and another one representing an additional ATPase activity originating from the SR and possibly also from other organelles. It was found that the Ca^{2+}-dependent ATPase activity could be well fitted by a sum of these two Hill curves. The Hill curve describing actomyosin ATP turnover was derived from the curve describing isometric force in saponin-skinned preparations, with a pCa for half-maximal activity (pCa_{50}) of 5.44 ± 0.07, and a coefficient of steepness (n_H) of 4.4 ± 0.4. The second Hill curve represented another Ca^{2+}-dependent ATPase activity with a pCa_{50} of 6.21 ± 0.09 and n_H of 3.5 ± 1.6. The contribution of the actomyosin interaction to the total Ca^{2+}-dependent ATPase activity in saponin-skinned muscle amounted to 82 ± 3%, and the contribution of the ATPase activity represented by the second Hill curve amounted to 18 ± 3%. We conclude from these data that, apart from actomyosin interaction, there is another Ca^{2+}-dependent ATPase activity present. This activity may reflect the ATPase activity of the SR Ca^{2+} pump.

To determine the contribution of the SR Ca^{2+} pump to the maximal Ca^{2+}-activated ATPase activity, we investigated the effects of 20 μM CPA, dissolved in 1% ethanol. It was found that maximal Ca^{2+}-activated ATPase activity in the presence of CPA was decreased to 79 ± 3% of the control value ($n = 12$). Isometric force decreased to 86 ± 2%. When 1% ethanol alone was added, ATPase activity

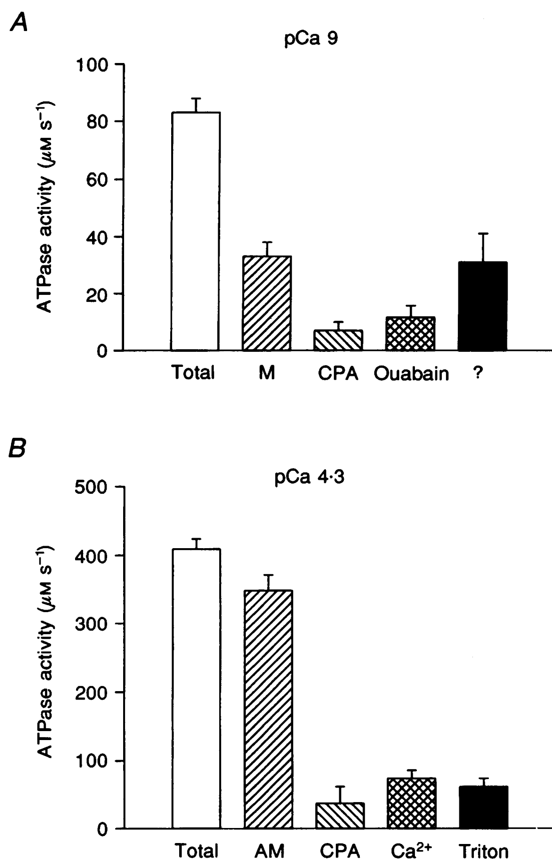

Figure 5. Summary of concurrent ATPase activities in relaxing solution (pCa 9, *A*) and activating solution (pCa 4·3, *B*)

A, total basal ATPase activity (Total), determined in saponin-skinned preparations; the contribution of myosin ATPase activity (M), determined in Triton-skinned preparations; basal ATPase activity that was inhibited by addition of 20 μM CPA (CPA); basal ATPase activity that was inhibited by addition of 1 mM ouabain (Ouabain); the remaining ATPase activity in relaxing solution that was removed by Triton but could not be blocked by CPA or ouabain (?). *B*, total maximal Ca^{2+}-activated ATPase activity (Total); contribution of the maximal Ca^{2+}-activated actomyosin ATPase activity (AM), determined in Triton-skinned preparations; the amount of maximal Ca^{2+}-activated ATPase activity that was suppressed by CPA (CPA); the contribution of the second Ca^{2+}-dependent ATPase activity to the overall ATPase activity (Ca^{2+}), determined by fitting the data with a sum of two Hill curves; the fraction of membrane-bound ATPase activity that could be abolished by Triton treatment (Triton).

decreased to 88 ± 3%, and isometric force decreased to 85 ± 1% ($n = 5$). Assuming that CPA and ethanol act independently, the net effect of CPA on maximal Ca^{2+}-activated ATPase activity thus amounts to a reduction of 9 ± 4% with respect to the control value.

Similar experiments were performed using thapsigargin, which is also known to be a specific inhibitor of the SR Ca^{2+} pump (Kijima *et al.* 1991; Kirby, Sagara, Gaa, Inesi, Lederer & Rogers; 1992). Preparations were allowed to incubate in 200 nM thapsigargin for 30–60 min. Thapsigargin was added from a 200 μM stock, dissolved in dimethyl sulphoxide (DMSO), yielding a final concentration of DMSO of 0·1% (v/v). When 0·1% DMSO alone was added, no significant changes in ATPase activity or force were observed. Addition of 200 nM thapsigargin had no significant effect on force (98 ± 3%) or ATPase activity (99 ± 1%, $n = 4$). Since the blocking of the Ca^{2+} pump by thapsigargin is probably an irreversible effect (Thomas & Hanley, 1994), the effects of thapsigargin were determined immediately following a control measurement. Thereafter the preparation was either discarded or underwent additional treatment with Triton for further measurements.

Another agent that is reported to be a potent inhibitor of SR ATPase activity is 2,5-di(*tert*-butyl)hydroquinone (TBQ; Thomas & Hanley, 1994; Westerblad & Allen, 1994). We measured the effects of 25 μM TBQ, dissolved in DMSO. ATPase activity was reduced to 86 ± 2% of the control value, whereas force was reduced to 92 ± 6% ($n = 5$).

Since the SR ATPase activity is not necessarily completely blocked by CPA, thapsigargin and TBQ, the maximal reduction in ATPase activity caused by these inhibitors provides a lower estimate for the maximal Ca^{2+}-activated ATPase activity of the SR. A maximal reduction of 9 ± 4% was observed when CPA was added, yielding a lower estimate of 37 ± 15 μM s^{-1}. Triton treatment showed that 15 ± 3% of the maximal Ca^{2+}-activated ATPase activity was membrane bound. We also showed the presence of a Ca^{2+}-dependent ATPase activity, which accounted for 74 ± 12 μM s^{-1}, or 18 ± 3% of the maximal Ca^{2+}-activated ATPase activity. These measurements therefore provide a lower limit of 37 ± 15 and an upper limit of 74 ± 12 μM s^{-1} for the maximal Ca^{2+}-activated ATPase activity of the SR.

The contribution of the Na^{+}–K^{+} pump to the maximal Ca^{2+}-activated ATPase activity was investigated by addition of 1 mM ouabain to the activating solution. This concentration is relatively high in comparison with the concentrations used in studies in intact preparations, and proved to result in a near-maximal effect on basal ATPase activity. However, after correction for the effect of ouabain on basal ATPase activity, no significant effect of ouabain on isometric force (97 ± 3%) or maximal Ca^{2+}-activated ATPase activity (97 ± 2%, $n = 7$) was observed.

The results of the experiments described above are summarized in Fig. 5. In this figure, the total maximal Ca^{2+}-activated and basal ATPase activity are shown. The relative contribution of the myofibrils and the putative contribution of the SR to the maximal Ca^{2+}-activated ATPase activity are also shown. The basal ATPase activity determined in Triton treated preparations is likely to be a basal myosin ATPase activity. This ATPase activity (40%), the activity that was depressed by ouabain (14%) and the activity that was depressed by CPA (8%) together amount to 62% of the basal ATPase activity in saponin-skinned preparations. The remainder of 38% (31 ± 10 μM s^{-1}) could not be ascribed to the myosin ATPase, the Na^{+}–K^{+} pump or the SR.

DISCUSSION

The goal of the experiments presented in this paper was to obtain an estimate for the maximal ATPase activity of the cardiac SR Ca^{2+} pump. In addition, it was found that several processes contribute to the basal ATPase activity in saponin-skinned preparations, which forms a relatively large fraction of the total ATPase activity.

Basal ATPase activity

Our experiments indicate that the basal ATPase activity constitutes a considerable fraction of the overall ATP turnover rate. Since the initial amount of basal ATPase activity (before measurements were performed) is estimated to be 189 ± 54 μM s^{-1}, these experiments indicate that basal metabolic activity may constitute about 25–35% of the total ATPase activity in cardiac muscle. Since mitochondrial activity was inhibited in these experiments by the addition of oligomycin and sodium azide, this value may even be larger *in vivo*. Such a relatively high value is in agreement with values for the metabolism in resting cardiac muscle that were reported earlier by several authors (e.g. Gibbs, 1978; Daut & Elzinga, 1988; Schramm *et al.* 1994). In rat cardiac muscle, values of one-third of the total cardiac metabolism have been reported for the resting metabolism (Loiselle & Gibbs, 1979). Although these values are in general agreement with the results presented here, it should be noted that they were obtained in intact papillary muscles and trabeculae. Because permeabilization of the sarcolemma by saponin could have an effect on basal metabolism, these results are not directly comparable.

Additional Triton treatment of saponin-skinned preparations showed that 40% of the basal ATPase activity is not membrane bound. This ATPase activity constitutes 8% of the total (i.e. basal and maximal Ca^{2+}-activated) ATP turnover in saponin-skinned preparations, and probably reflects unregulated myosin ATPase activity in the myofibrils. This value is in agreement with results obtained earlier by us in Triton-skinned preparations (Ebus *et al.* 1994) and in dog cardiac myofibrils (Solaro, Wise, Shiner & Briggs, 1974).

About 14% of the basal ATPase activity was inhibited by the addition of ouabain, which indicates that the Na^+-K^+ transporter contributes to the basal ATPase activity. Although the concentration of ouabain used in this study is relatively high, we cannot rule out the possibility that complete inhibition of the Na^+-K^+ transporter is not achieved under our experimental conditions. It can be noted, however, that this relative contribution compares well with results obtained by Schramm *et al.* (1994) in intact trabeculae from guinea-pig at 37 °C.

A small amount of basal ATPase activity (7 μM s^{-1}) was inhibited by CPA. It is not clear whether this activity reflects basal SR ATPase activity, active at low (pCa 9) calcium concentrations. However, this value is so small that identifying its exact origin would be extremely difficult.

When the basal ATPase activity in saponin-skinned preparations of 83 μM s^{-1} is corrected for the decrease observed in the presence of Triton X-100, ouabain and CPA, a relatively large amount of basal ATPase activity (38%, or 31 μM s^{-1}) cannot be accounted for by the myosin, Na^+-K^+ or possible basal SR ATPase activity. This remaining activity was abolished by Triton treatment, and therefore must be membrane bound. Since mitochondrial activity was inhibited by the addition of oligomycin and azide, it is unlikely that this ATPase activity originates from the mitochondria. This indicates that 31 $\pm$ 10 μM s^{-1} cannot be accounted for by SR, AM, or Na^+-K^+ ATPase activity.

Given the morphology of cardiac muscle, in which sarcolemma and the transverse tubules (T-tubules) constitute a relatively large membrane area, this Ca^{2+}-independent ATPase activity could be a Ca^{2+}-independent Mg^{2+}-ATPase located in the T-tubules and/or the sarcolemma. Several authors have reported an active, Ca^{2+}-independent Mg^{2+}-ATPase in isolated vesicles from T-tubules (Moulton, Sabbadini, Norton & Dahms, 1986) and in microsomes isolated from several types of tissue from rat (Beeler *et al.* 1985). Beeler *et al.* have suggested that this ATPase might be an ectoenzyme, which would catalyse the first step of hydrolysis of nucleotide-5′-triphosphates. Moulton *et al.* (1986) suggested that the ATPase would support formation of proton gradients. However, the specific function of this ATPase has not yet been satisfactorily explained.

Since saponin perforates the sarcolemma by binding to cholesterol (Endo & Iino, 1980), the sarcolemma remains largely present, albeit in a permeabilized condition. It is most likely, therefore, that the Ca^{2+}-independent ATPase activity being lost during repeated measurements in relaxing solution are ATPases attached to fragments of the sarcolemma and/or T-tubules that are partly dissolved by the saponin treatment of the preparation. This view is supported by the observation that the decrease of basal ATPase activity was dependent upon fibre diameter: thicker preparations required more measurements in relaxing solution before basal ATPase activity reached a steady level. Beeler, Gable & Keffer (1983) have shown that the Mg^{2+}-ATPase of the T-Tubules in membrane fractions is inhibited by ATP, and decreases exponentially with time to a low steady state, with a half-time of about 4 min. Since preparations spent approximately 1·5 h in solutions containing an ATP-regenerating system, inactivation by ATP cannot be an explanation for the decrease observed in our preparations.

In conclusion, we find that the basal ATPase activity in saponin-skinned cardiac trabeculae is relatively high. Putative candidates for this activity are: myosin (about 40%), Na^+-K^+ exchange (about 15%), SR (about 8%), T-tubules (about 35%). Since the possibility that the effects of ouabain and CPA on the basal ATPase activity are submaximal cannot be excluded, the value of 35% for the putative ATPase activity of the T-tubules therefore constitutes an upper limit for this activity.

Maximal Ca^{2+}-activated ATPase activity

In order to determine the contribution of the maximal SR Ca^{2+} transporting ATPase to the overall ATP turnover, we studied the effects of Triton treatment and of specific inhibitors of the SR Ca^{2+} ATPase activity. Furthermore, we investigated the Ca^{2+} dependence of the Ca^{2+}-activated ATPase activity.

It might be argued that 5 mM caffeine is not sufficient to ensure continuous and maximal release of Ca^{2+} from the SR. Experiments with 25 mM added caffeine showed a decrease in maximal Ca^{2+}-activated ATPase activity and force in comparison with the values measured in the presence of 5 mM caffeine. Although the decrease in ATPase activity was slightly less than the decrease in force, the difference was not significant. This indicates that there is no relative increase in ATPase activity at higher caffeine concentrations. However, since high caffeine concentration also influences the myofibrillar apparatus, we cannot completely rule out the possibility that it increases the rate of ATP turnover by the SR Ca^{2+} pump. Ca^{2+} release from the SR is further aided by the fact that the measurements were performed under maximal Ca^{2+} activation (pCa 4·3). The release of Ca^{2+} from the SR would therefore be facilitated by the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR). Furthermore, it can be noted that neither addition of 0·1% (v/v) Triton X-100 nor 20 μM A23187 increased maximal Ca^{2+}-activated ATPase activity. Although it has been suggested that caffeine may inhibit the SR Ca^{2+} pump in vesicles (Mészáros & Ikemoto, 1985) and in intact mouse skeletal muscle (Allen & Westerblad, 1995), our findings indicate that this is not the case in saponin-skinned cardiac muscle. We are therefore confident that the combination of 5 mM caffeine and high [Ca^{2+}] is sufficient to ensure adequate Ca^{2+} release from the SR. The mechanism of CICR has been shown to be present in cardiac muscle for Ca^{2+} concentrations of about pCa 7 and higher (Fabiato &

Fabiato, 1975), but might be less effective at low Ca^{2+} concentrations. However, the uptake of Ca^{2+} by the SR at these concentrations is also likely to be decreased, as our experiments indicate. Since the time that the preparations spent in solutions containing Ca^{2+} and caffeine was relatively short, we do not expect that Ca^{2+} accumulation in the SR impairs the rate of Ca^{2+} uptake.

Our experiments show that the maximal Ca^{2+}-activated ATPase activity is reduced by 9% when CPA is added. Thapsigargin and TBQ proved to be less effective under these experimental conditions. Although all these agents have been reported to be potent inhibitors of the SR Ca^{2+} pump in cardiac myocytes (Seidler *et al.* 1989; Baudet, Shaoulian & Bers, 1993; Thomas & Hanley, 1994), they may be less effective in multicellular preparations, such as the trabeculae used in this study (cf. Baudet *et al.* 1993). Experiments by Du, Ashley & Lea (1995) showed that the force response in caffeine-induced Ca^{2+} release in trabeculae from rat was half-maximal at 35·2 μM CPA after 18–24 min of loading. It is possible, therefore, that CPA may not have completely inhibited all the Ca^{2+} pumps in our preparations. CPA inhibition of the SR Ca^{2+} pump is [ATP] dependent (Seidler *et al.* 1989), and incomplete inhibition thus may be associated with the [MgATP] (5 mM) in the solutions. Therefore, the value of 9% depression of maximal Ca^{2+}-activated ATPase activity represents a lower boundary for the maximal SR Ca^{2+} pump activity.

An upper limit is provided by the Ca^{2+} dependence of saponin-skinned preparations, which showed the presence of a Ca^{2+}-dependent ATPase activity of 18 ± 3% of the total ATPase activity, and by the experiments with Triton, which showed that 15 ± 2% of the maximal Ca^{2+}-activated activity was membrane bound. These values are compatible with measurements of heat production in guinea-pig ventricular muscle by Schramm *et al.* (1994). These authors report a contribution of the SR of 15% to the contraction related heat production during twitches at 37 °C.

The Ca^{2+} dependence of ATPase activity in saponin-skinned muscle also shows that the SR Ca^{2+} pump is activated at relatively low Ca^{2+} concentrations. Our results indicate that the Ca^{2+} pump is half-maximally active at pCa 6·2, whereas the actomyosin ATPase activity is half-maximal at a Ca^{2+} concentration which is almost an order of magnitude larger (pCa 5·4). The coefficient of steepness, n_H, is relatively small, indicating that the Ca^{2+} pump is submaximally active over a wide range of calcium concentrations.

These results are quite different from results found earlier in skeletal muscle (Stienen, Zaremba & Elzinga, 1995). The basal ATPase activity in fast skeletal muscle comprises about 1–2% of the total ATP turnover, which is considerably less than the Ca^{2+}-independent ATPase activity in cardiac muscle (17% of the total ATP turnover rate) found in this study. On the other hand, whereas the SR Ca^{2+} transporting ATPase activity in cardiac muscle is found to amount to 9–18% of the total Ca^{2+}-activated ATPase activity, this value amounts to about 25 and 40% in fast and slow skeletal muscle, respectively (Stienen *et al.* 1995). These relative differences are in general agreement with values obtained in canine cardiac microsomes and rabbit fast skeletal microsomes by Shigekawa, Finegan & Katz (1976). These authors report a maximum velocity of ATPase activity of 0·45 μmol mg^{-1} min^{-1} in cardiac microsomes and 1·60 μmol mg^{-1} min^{-1} in skeletal microsomes, i.e. a 3·5-fold difference.

Our results show a maximal rate of ATP turnover of the cardiac SR Ca^{2+} transporter of 37–74 μM s^{-1}. Experiments in rat ventricular cells by Hove-Madsen & Bers (1993) showed a Ca^{2+} uptake rate of 37–49 nmol (mg $protein)^{-1}$ min^{-1}. These authors assumed a value of 70 mg cell protein (g wet weight of $ventricle)^{-1}$, and the Ca^{2+} uptake rate of 37–49 nmol (mg $protein)^{-1}$ min^{-1} thus amounts to 43–57 μM Ca^{2+} s^{-1}. Assuming a stoichiometry of 2 : 1, this amounts to 22–29 μM ATP s^{-1}. Similarly, Balke, Egan & Wier (1994) found a maximal Ca^{2+} uptake rate of 31 nmol mg^{-1} min^{-1}, or about 18 μM ATP s^{-1} in intact rat cardiac cells (room temperature). These values are about a factor of 2 smaller than the maximal SR ATPase activity we find in saponin-skinned preparations.

A complicating factor in the comparison between the results found by these authors in myocytes and our results in skinned trabeculae is the increase in volume which occurs upon skinning preparations. Since the outer diameter of the preparations used in this study increased by about 10–20%, this would indicate that the volume of intact preparations would be 1·2–1·5 times smaller, and therefore the ATPase activity per volume would be about 1·2–1·5 times higher than the values found in this study. This effect further accentuates the difference between our results obtained in saponin-skinned muscle and the results found by Hove-Madsen & Bers (1993) and Balke *et al.* (1994) in cardiac myocytes.

The smaller value of ATP hydrolysed found by these authors might be explained if the coupling ratio between Ca^{2+} transported and ATP hydrolysed by the Ca^{2+} transporting ATPase is smaller than the optimal value of 2. Several authors have pointed out that the coupling ratio between Ca^{2+} transported and ATP hydrolysed is not necessarily 2 : 1 (e.g. Galina & de Meis, 1991; Korge & Campbell, 1994). Reduction of this ratio is mainly observed in systems in which ADP is present, and/or with elevated SR luminal Ca^{2+} levels. On the other hand, Inesi & de Meis (1989) found that in the presence of an ATP-regenerating system (phosphocreatine based or PEP based, as is the case in our experiments) the coupling ratio closely approximated 2 in SR vesicles from rabbit skeletal muscle.

In summary, we find that the Ca^{2+}-dependent ATP turnover in saponin-skinned cardiac muscle can be well

described as the sum of the ATP utilization by the myofibrils and by the ATPase activity of the SR Ca^{2+} transporting ATPase. This latter ATPase activity is half-maximal at pCa 6·2, and constitutes 18%, or 74 ± 12 μM s^{-1} of the total ATPase activity in rat cardiac muscle. Experiments with CPA showed that at least 37 ± 15 μM s^{-1} of the total activity can be ascribed to the SR Ca^{2+} ATPase. We therefore estimate the maximal Ca^{2+} ATPase activity of the SR Ca^{2+} pump to range between 37 and 74 μM s^{-1}.

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