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Carbonic anhydrase in skeletal and cardiac muscle from rabbit and rat

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We have studied the distribution of carbonic anhydrases (CA) in several skeletal muscles of the hindlimb of rabbits and rats and in cardiac muscle of the rabbit. To remove erythrocyte CA, hindlimbs and hearts were thoroughly perfused with dextran solution, and the effectiveness of the perfusion was in most cases assessed by determining the contamination of the muscles with radioisotopes that had been used to label the erythrocytes before the perfusion was started. We observed three forms of CA: (1) cytosolic (sulphonamide-resistant) CA III; (2) a cytosolic sulphonamide-sensitive CA, probably isoenzyme II; (3) a membrane-bound form that was extracted from the particulate fraction using Triton X-100. These CA isoforms were distributed as follows. (1) CA III is located in the cytoplasm of slow, oxidative skeletal muscles and is absent from or low in fast skeletal and cardiac muscle; this holds for rabbits and rats and is identical with the pattern previously described for several other species. (2) The cytosolic sulphonamide-sensitive CA is present in fast rabbit muscles and absent from slow muscles of this species. In contrast, all skeletal muscles of the rat studied here lack, or possess only very low, activity of this isoenzyme. (3) The membrane-bound form of CA is present in all rabbit muscles studied; its activity appears somewhat higher in fast than in slow skeletal muscles. (4) Cardiac muscle constitutes an exception among all striated muscles of the rabbit as it possesses no form of cytosolic CA but a high activity of the membrane-bound form.

INTRODUCTION

It has been demonstrated that at least three types of carbonic anhydrase (CA) are present in skeletal muscles: (1) a sulphonamide-resistant isoenzyme, CA III, which appears to be present in the cytosol of slow oxidative fibres in red skeletal muscles (Holmes, 1977; Shima *et al.*, 1983; Jeffery *et al.*, 1986; Väänänen *et al.*, 1986; Edwards, 1991); (2) a sulphonamide-sensitive cytosolic isoenzyme which seems to be identical with erythrocyte CA II (Siffert & Gros, 1982); (3) a membrane-bound form which is present in the sarcolemma (Dermietzel *et al.*, 1985; Geers *et al.*, 1985; Wetzel & Gros, 1988, 1990) and in the sarcoplasmic reticulum (Bruns *et al.*, 1986). In cardiac muscle no cytosolic CA has been detected (Holmes, 1977; Moynihan, 1977; Carter *et al.*, 1982). Whether a membrane-bound CA is present in cardiac muscle is not clear; there is indirect evidence for the presence of an extracellular CA in heart muscle from surface pH measurements (de Hemptinne *et al.*, 1987), whereas results from studies of the space of distribution of $H^{14}CO_3$ seem to indicate the absence of such a CA in heart muscle (Zborowska-Sluis *et al.*, 1975). No direct evidence for a membrane-bound CA in heart has been reported so far. The bulk of the present studies has been done with rabbit muscles, but some complementary studies have been performed with rat muscles.

We have investigated the distribution of these three isoenzymes in various skeletal muscles which differ with respect to their fibre-type composition and myoglobin concentration. We have confirmed the absence of a cytosolic CA from heart muscle, but show that there is a membrane-bound CA in the heart. Our data suggest that the CA II-like cytosolic CA is confined to fast fibres of the rabbit and is only present in fibres that do not contain CA III, but apparently is absent from all fibre types in the rat. In addition, we report some enzymic properties of the two cytosolic CAs.

METHODS

Preparation of muscle tissue homogenate

Measurements of the activity of sulphonamide-sensitive CA were performed on homogenates of blood-free perfused muscle tissue from the hindlimbs or heart of adult rabbits (New Zealand White, 3–4 kg). After anaesthetization of the rabbits with Ketamin (50 mg/kg; WDT, Hannover, Germany)/Rompun (0.5 ml/kg; Bayer, Leverkusen, Germany), their hindlimbs were artificially perfused with a dextran solution [0.9% (w/v) NaCl/3% (w/v) dextran, M_r 60000] via a catheter inserted into the femoral artery until the effluent leaving the catheter in the femoral vein was free of erythrocytes. After this perfusion procedure, individual muscles from the calf only were taken for further study. When muscles from both the thigh and the calf were to be used (for experiments with pooled muscles), perfusion was accomplished through a catheter inserted into the abdominal aorta distal to the renal artery, and the effluent was collected from the caval vein. Rabbit hearts were perfused through catheters inserted into the ascending aorta, the tip of the catheter pointing toward the aortic valves. In order to obtain perfused muscles from the hindlimbs of rats (Wistar), a catheter was inserted through the incised left chamber of the heart into the ascending aorta, where it was fixed. Through this catheter the whole body of the rat was perfused, the effluent leaving the opened right atrium.

To be able to estimate the content of erythrocyte CA in the perfused rabbit muscle, the erythrocytes of the circulating blood were labelled with either ^{51}Cr or $K^{14}CNO$ about 1 h before the perfusion was started. ^{51}Cr labelling was done as described previously (Gros *et al.*, 1980). Labelling with $K^{14}CNO$ was performed as follows. Approx. 20 ml of erythrocytes were withdrawn from the circulation on the day before the perfusion experiment, washed in 0.9% NaCl/5 mM-glucose, and incubated

Abbreviation used: CA, carbonic anhydrase; SO, slow-twitch oxidative; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative-glycolytic.

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overnight in 20 ml of the same solution containing, in addition, 125 μCi of $K^{14}CNO$ (52 mCi/mmol; Amersham Buchler, Braunschweig, Germany). On the next day the erythrocyte suspension was centrifuged (5000 rev./min, 10 min) and the pelleted erythrocytes were suspended in 0.9% NaCl/5 mM-glucose. This washing step was repeated several times to remove free $K^{14}CNO$ until the radioactivity in the supernatant reached a constant low level which was believed to be due to a very small degree of haemolysis of the labelled erythrocytes rather than to free $K^{14}CNO$. The final erythrocyte pellet was suspended in the plasma fraction of the original blood sample, and injected into the jugular vein of the rabbit. After 1 h the perfusion of the muscle tissue was begun. By comparing the radioactivity of the perfused muscles after perfusion with the radioactivity of the circulating blood before the perfusion, the contamination of the muscle tissues with residual erythrocytes was evaluated. Knowing the CA activity of the erythrocytes, the CA activity of the muscle homogenate supernatant due to residual erythrocytes could be estimated. Unlike in rabbits, the effectiveness of the erythrocyte removal by perfusion was not controlled by using radioisotopes in the case of whole-body-perfused rats.

At the end of the perfusion, individual muscles were excised and, after homogenization, used for the determination of sulphonamide-sensitive CA activity by a micromethod (see below). For measurements of the sulphonamide-resistant CA, CA III, perfusion was omitted in some cases, but muscles were also excised and homogenized individually. For CA activity determinations performed in the stopped-flow apparatus (see below), perfused red and white muscles were separately pooled because rather large amounts of homogenate were required for these measurements. Only muscles appearing completely white were classified as 'white' while all other muscles, whether darkly red or only reddish, were classified as 'red'. For homogenization, muscles were cut into small pieces, frozen in liquid nitrogen, and the frozen tissue pieces were pulverized in a Mikro-Dismembrator (Braun, Melsungen, Germany). After thawing, the homogenate was centrifuged at 100000 *g* for 1 h (TGA 50 ultracentrifuge; Kontron, Munich, Germany). The supernatants were frozen and kept in liquid nitrogen until used for determination of CA activity.

To assess the activity of the CA associated with the membrane fraction of the muscles, the pellets obtained in the above ultracentrifugation were suspended in an approx. 10-fold volume of 0.9% NaCl, filtered through a double layer of cheesecloth and then centrifuged again at 100000 *g* for 1 h. The washing procedure was repeated at least four times until the CA activity in the supernatant was less than 1–2 units. The final pellet was incubated overnight in 1.5 vol. of 2% (w/w) Triton X-100. Thereafter, this suspension was centrifuged at 500 *g* for 10 min. The supernatant containing extracted membrane proteins was used for CA and other determinations.

Determination of CA activity

CA activity in the pooled muscle homogenates was determined as described by Siffert & Gros (1982) by using a rapid-reaction stopped-flow apparatus equipped with a pH-sensitive glass electrode (Crandall *et al.*, 1971; Gros *et al.*, 1976). Briefly, enzyme activities were determined from measurements of the rate of CO_2 hydration using a 30 mM-imidazole/HCl buffer with 0.15 M-NaCl (pH 7.2). The supernatants were diluted 1:3 to 1:10 in this CO_2-free buffer. The diluted supernatants were then rapidly mixed at 25 °C with CO_2 solutions (0.15 M-NaCl equilibrated with various CO_2/N_2 mixtures) in the stopped-flow apparatus. From the initial slope of the recorded pH trace and the buffer capacity of the solutions, the initial rate of CO_2 hydration was calculated. Subtracting the independently determined uncatalysed rate, the rate of CO_2 hydration due to enzyme catalysis was obtained. Enzyme activity was expressed as the ratio of catalysed rate over uncatalysed rate of hydration. This method allowed determination of K_m and $V_{max.}$ values, and for this purpose the measurements were repeated at different initial CO_2 concentrations (between 1.5 and 12 mM) and the data plotted according to Lineweaver–Burk.

CA activity in individual muscles was determined using the micromethod of Maren *et al.* (1960) as modified by Bruns *et al.* (1986). In this method the time necessary for Phenol Red to change its colour from red to yellow after addition of an alkaline buffer is determined in the presence of 100% CO_2 at 0 °C. One unit of CA activity is defined as the concentration of enzyme required in the final assay volume (0.4 ml) to halve the time necessary in the absence of CA. Inhibition curves with acetazolamide (Lederle, Munich, Germany) were determined using enzyme concentrations giving an activity of about 2 units in the absence of inhibitor.

Activity of CA III in homogenates of both perfused and unperfused muscles was measured in the presence of 10 μM-acetazolamide, a concentration that completely inhibited sulphonamide-sensitive CAs but did not affect CA III activity. In cases where the activity of both types of CA was to be determined, the activity of the sulphonamide-sensitive CA was obtained by subtracting the activity of CA III from the total activity of the sample as measured in the absence of inhibitors.

To compare the activities obtained with the two methods, preparations of soleus homogenates containing CA III, and lysed erythrocytes of rabbits containing the sulphonamide-sensitive enzyme, were studied with both the stopped-flow and the micromethod. Since in the stopped-flow experiments CA I was virtually completely inhibited by the high concentration of Cl^- ions present (Maren *et al.*, 1976), and because in the micromethod a high concentration of bicarbonate should have the same effect on CA I (Maren *et al.*, 1976), essentially only CA II was measured with both methods in the case of the rabbit erythrocytes. In the case of the soleus homogenate, a high concentration of acetazolamide was added which left only CA III uninhibited. With the micromethod (0 °C, pCO_2 98.7 kPa), CA III activity and CA II activity were higher by a factor of 1.7 and 1.2 respectively than the activities determined with the stopped-flow method (25 °C, pCO_2 after mixing 9.6 kPa). It may be noted that, owing to the presence of high concentrations of anions, CA III activity is decreased to a defined degree in both methods, while CA II activity should be virtually unaffected.

Analytical procedures

Protein concentrations were determined using a biuret method modified according to Metz & Reinert (1974) but using 0.5% Triton X-100 for sample solubilization. Radioactivities were measured in a Tri-Carb liquid-scintillation spectrometer (C1425; Packard Instruments) after combustion of the samples in an oxidizer (B360; Packard). Myoglobin concentrations in the supernatants of homogenates were estimated according to the method of Reynafarje (1963) from the absorbances read at 539 nm and 570 nm. To be able to estimate average enzyme activities in the tissue from the activities measured in the homogenate supernatants, the Cl^- space of the homogenate pellets was determined by repeated washing of the pellet and determination of Cl^- in the original supernatant and in the supernatants obtained from the washing steps. Assuming the total Cl^- space of the homogenate (volume of initial supernatant plus Cl^- space of the initial pellet) to be identical with the space available for a cytosolic enzyme in that homogenate, it was estimated that an activity found in the supernatant must be multiplied by 0.7 to give the average activity in the fresh tissue.

RESULTS

Sulphonamide-sensitive CA and CA III in supernatants of pooled red and white skeletal muscle homogenates

Figs. 1(*a*) and 1(*b*) show inhibition curves of the CA activity in the supernatants from pooled white and red muscles respectively, as measured in the rapid-pH stopped-flow apparatus. Contamination of these muscles with erythrocytes was extremely low, 0.001–0.01 % (w/w), such that no more than 1 % of the CA activities measured in the supernatants (see Table 1) should originate from residual erythrocyte CA. As already reported (Siffert & Gros, 1982), in the case of white muscle (Fig. 1*a*) a monophasic inhibition curve is obtained which can be represented in terms of one value of CA concentration, $[E_{tot.}]$, and one value of the inhibition constant, K_i. Since the known cytosolic isoenzymes CA I, CA II and CA III of rabbits have markedly different K_i values towards ethoxzolamide (Siffert & Gros, 1982, and Table 1 of this study), this is compatible with only one isoenzyme being, at least functionally, present in white muscle supernatant. In contrast, a biphasic inhibition curve is obtained for pooled red muscle supernatant (Fig. 1*b*). At an ethoxzolamide concentration of 50 nM, a concentration at which the CA activity of white muscle supernatant is almost completely suppressed, there is still substantial residual activity. This 'sulphonamide-resistant' CA activity does not begin to decrease before the inhibitor concentration is increased by two orders of magnitude. Owing to the limited solubility of ethoxzolamide, full inhibition cannot be achieved with this drug but it is obtained in Fig. 1(*b*) with the more soluble acetazolamide. We conclude that in pooled red muscle supernatant two CA isoenzymes occur, one with a high sensitivity towards ethoxzolamide, sulphonamide-sensitive CA, which is similar to that observed in white muscle supernatant, and one whose extremely low sensitivity towards the sulphonamide allows us to identify it as CA III (Holmes, 1976, 1977; Koester *et al.*, 1977; Carter *et al.*, 1978).

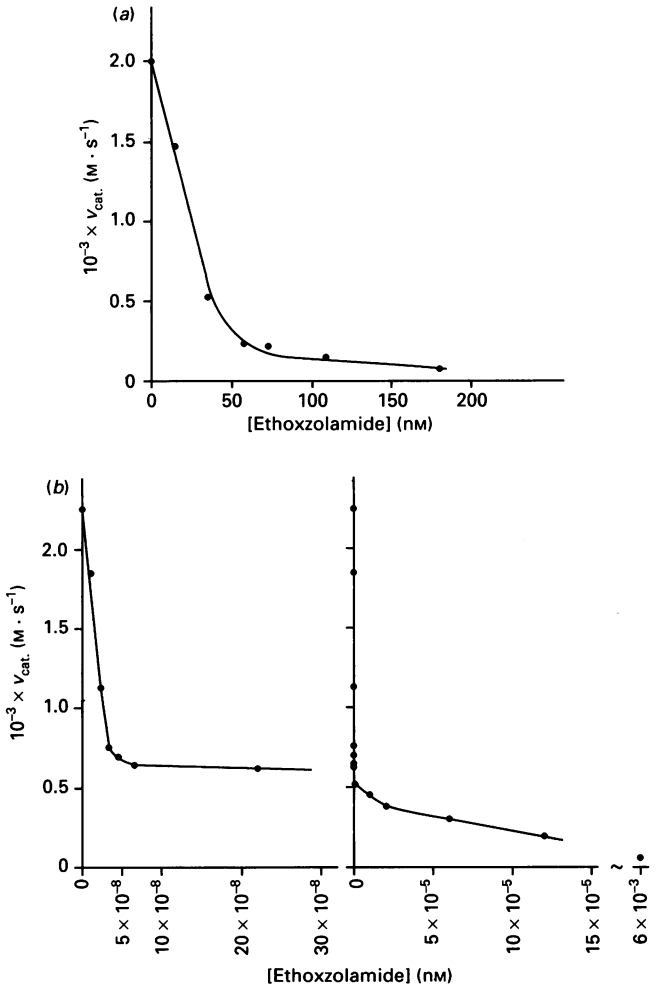

Fig. 1. Inhibition curves of supernatant from pooled rabbit skeletal muscles

The catalysed rate of CO_2 hydration, $v_{cat.}$, is plotted versus total concentration of ethoxzolamide. The measurements were performed in a rapid-pH stopped-flow apparatus (0.15 M-NaCl/0.015 M-imidazole, pH 7.2, initial pCO_2 after mixing 9.6 kPa, 25 °C). Contamination with erythrocyte CA is negligible. (*a*) Inhibition curve of diluted supernatant from pooled perfused white muscles from rabbit hindlimb. The data points are fitted best with K_i of 0.9 nM. (*b*) Inhibition curves of diluted supernatant from pooled perfused red muscles from rabbit hindlimbs. The pool of muscles used encompasses muscles containing SO as well as FOG fibres. The data points are best described in terms of two isoenzymes, one with K_i = 0.4 nM and one with K_i = 60 μM. The data point at a sulphonamide concentration of 6 mM in the right-hand part of (*b*) does not refer to ethoxzolamide but to the more soluble acetazolamide. The curve illustrates that it is possible to study the sulphonamide-resistant isoenzyme (CA III) by using intermediate concentrations of inhibitor completely inhibiting the sulphonamide-sensitive CA but leaving the sulphonamide-resistant form unaffected. This principle is used throughout this study.

Table 1. Properties of CAs in supernatants of pooled perfused white and red rabbit hindlimb skeletal muscle homogenate

All measurements were done in a rapid-pH stopped-flow apparatus at 25 °C, pH 7.2, (0.15 M-NaCl/0.015 M-imidazole). From inhibition curves such as those of Fig. 1 the inhibition constants, K_i, for ethoxzolamide and the total enzyme concentration, $[E_{tot.}]$, were estimated. By measuring the catalytic rate of CO_2 hydration at various CO_2 concentrations, the Michaelis constant for CO_2, K_m and (from $V_{max.}$ together with $[E_{tot.}]$) the turnover number of the enzyme, $k_{cat.}$, were determined. Activity refers to CA activity as defined in the Methods section for pCO_2 = 9.6 kPa and 25 °C. All values refer to undiluted supernatants from homogenates of pooled muscles: white hindlimb muscles [sulphonamide-sensitive CA (SS-CA) only] and red hindlimb muscles (sulphonamide-sensitive CA and the sulphonamide-resistant CA III) (n = 2). Data for white muscles came from Siffert & Gros (1982).

	Supernatants from pooled muscles:		
	White SS-CA	Red SS-CA	Red CA III
K_i (ethoxzolamide) (nM)	0.6	0.5	60000
K_m (mM)	3.2	2.9	1.9
$k_{cat.}$ (s^{-1})	90000	130000	35
Activity (units)	160	250	50

Table 1 gives a compilation of inhibition and kinetic constants characterizing the CAs of pooled white and red muscle supernatants. The values for K_i and $[E_{tot.}]$ were derived as described previously (Siffert & Gros, 1982). In the case of red muscle supernatant, the two phases of the inhibition curves were analysed separately to yield K_i and $[E_{tot.}]$ for sulphonamide-sensitive CA as well as CA III. $V_{max.}$ and K_m for CA III were derived from measurements performed at various CO_2 concentrations in the presence of 10 μM-acetazolamide. The same parameters for sulphonamide-sensitive CA were calculated from data obtained in the absence of inhibitors. Although under the latter conditions sulphonamide-sensitive CA as well as CA III contribute to the total CA activity, the values of $V_{max.}$ and K_m derived from these data very closely represent those for sulphonamide-sensitive CA of red muscle supernatant. Using the

Table 2. Sulphonamide-sensitive CA in supernatant of tissue homogenate

From the fraction of erythrocytes in the supernatant (w/w) the CA II activity (A) due to erythrocyte CA was estimated, assuming $A = 20000$ for rabbit erythrocyte CA. Sulphonamide-sensitive activity was obtained as the difference between total and sulphonamide-resistant activity. One unit indicated the concentration necessary to cut the reaction time of CO_2 hydration under the condition of Maren *et al.*'s (1960) assay down to one half. Given are mean values ± S.D.; n is the number of animals whose muscles were studied; $n = 2$ if not stated otherwise. n.s., not significant from zero. Red thigh muscle includes vastus intermedius/semimembranosus proprius.

Muscle	Erythrocytes/ tissue (%, w/w)	Activity of sulphonamide-sensitive CA: Due to erythrocytes (units)	Activity of sulphonamide-sensitive CA: Total CA in supernatant (units)	Activity of sulphonamide-sensitive CA: Due to muscle tissue (units)	Specific CA activity (units/ml per mg of protein)
Rabbit					
Heart ($n = 6$)	0.26±0.23	52±46	35±11	n.s.	–
Soleus ($n = 3$)	0.06±0.05	12±11	16±4	n.s.	–
Red thigh muscle	0.17	35	86	51	1.1
Tibialis anterior	0.02	5	386	381	4.3
Extensor digitorum longus	0.03	6	248	243	3.3
Gastrocnemius, white	0.03	6	285	279	5.4
Gastrocnemius intermedius	0.02	5	165	160	2.6
Gracilis	0.01	2	233	231	6.7
Rat					
Soleus			19		
Vastus intermedius			16		
Semimembranosus ($n = 3$)			14±8		
Tibialis anterior ($n = 3$)			18±20		
Extensor digitorum longus ($n = 3$)			17±11		
Gastrocnemius, white ($n = 3$)			9±8		
Gastrocnemius, red			56		
Vastus lateralis ($n = 3$)			9±9		

constants of Table 1 it can be shown that the presence of CA III in these measurements introduces an error in $V_{max.}$ and K_m for sulphonamide-sensitive CA of no more than 20% and 10% respectively. The turnover numbers, $k_{cat.}$, given in Table 1 were calculated as $V_{max.}/[E_{tot.}]$.

Sulphonamide-sensitive CA and CA III in individual skeletal muscles and in cardiac muscle

Table 2 gives activities of sulphonamide-sensitive CA found in the homogenate supernatants of blood-free perfused muscle tissues. It is also shown that only 0.01–0.26% (w/w) erythrocytes were present in the rabbit muscles after perfusion. Assuming the CA activity of rabbit erythrocytes to be 20000 (Bruns *et al.*, 1986), the CA II activity due to residual erythrocytes remaining in the muscle after perfusion could be calculated as shown in Table 2. In the case of heart muscle and red soleus muscle the CA activity measured in the supernatant can be fully explained in terms of contaminating erythrocytes. Thus no cytosolic CA is detectable in the heart, in agreement with our own preliminary report (Gros *et al.*, 1980) or in the red soleus muscle, and very little activity is detectable in the combined red muscles vastus intermedius/semimembranosus proprius. Unlike these slow muscles, the various fast and mixed muscles listed in Table 2 for the rabbit have high activities of sulphonamide-sensitive CA, which agrees with the results from pooled white muscle homogenate.

Table 2 also gives the sulphonamide-sensitive CA activities found in the supernatants of thoroughly perfused hindlimb muscles of the rat. Although the presence of residual erythrocytes was not quantified in this case, their values, which place an upper limit to any sulphonamide-sensitive CA of the muscles themselves, are very low. It appears very likely that these CA activities represent CA of residual erythrocytes and that rat muscles, in contrast with fast rabbit muscles, do not possess a cytosolic sulphonamide-sensitive CA at all. In contrast, the sulphonamide-resistant CA III is present in both species, at the highest concentration in slow red muscles such as soleus (Table 3).

Sulphonamide-sensitive CA activity in the particulate fraction of skeletal and cardiac muscles

Table 4 shows that a CA activity that is sensitive to sulphonamides can be extracted from the membrane fraction of muscle homogenates. The remaining contamination with soluble CA was estimated from the CA activity in the supernatant of the last pellet washing; it never exceeded a value of 2 units. On treatment of the pellets with Triton X-100 and subsequent centrifugation, considerable CA activity appeared in the supernatants of cardiac as well as all skeletal muscles studied. Since microscopic inspection of the suspensions of untreated pellets did not show any intact cells, it is unlikely that this CA originates from the cytoplasm of cells that had remained intact during homogenization and were made leaky by the exposure to Triton X-100. We conclude therefore that this CA is associated with membranes or organelles. The amount of extractable membrane-bound CA varies markedly with muscle type, heart and masseter exhibiting the highest CA activities in the Triton X-100 extracts.

The inhibition constants, K_i, for acetazolamide of the CA of particulate fractions are quite similar for the various muscles (73–94 nM) and are clearly greater than those obtained by us for

Table 3. CA III activity and myoglobin concentration in supernatants of skeletal muscle homogenates from rabbit and rat

Values are given as means ± S.D. (*n* is the number of animals whose muscles were studied). 'Abdominal muscle' consists of M. obliquus internus and M. transversus abdominis only. CA III activity was determined in the presence of 10 μM-acetazolamide using Maren *et al.*'s (1960) micromethod.

Muscle	[Myoglobin] (mM)	*n*	CA III activity (units)	*n*
Rabbit				
Psoas	0.02	1	13	1
Tibialis anterior	0.06±0.02	13	10±3	12
Extensor digitorum longus	0.07±0.02	13	13±2	12
Abdominal muscle	0.09	1	170	1
Diaphragm	0.13	1	16	1
Vastus intermedius	0.19	1	372±36	5
Semimembranosus proprius	0.20	1	403	1
Masseter	0.21	2	< 1	3
Soleus	0.24±0.04	12	398±60	19
Rat				
Abdominal muscle	0.18	1	532	1
Diaphragm	0.25	1	130	1
Soleus	0.25	1	1119±272	14
Tibialis anterior	–	–	71±18	14
Extensor digitorum longus	–	–	28±13	12
Vastus intermedius	–	–	305±66	7
Gastrocnemius	–	–	58	2
Gastrocnemius, red	–	–	152	1

the supernatant of white gastrocnemius (28 nM) and by Bruns *et al.* (1986) for lysed rabbit erythrocytes (17 nM) and for pure bovine CA II (17 nM). As the CA activity of rabbit erythrocytes is almost entirely constituted by CA II (Siffert & Gros, 1982; see also above), the latter two values both refer to CA II. Thus the K_i values of the CA extracted from the particulate fraction appear to be three to five times higher than that for cytosolic CA II.

DISCUSSION

Contamination of the homogenate with erythrocytes

The conclusions drawn in this study are based on the assumption that the CA we find in muscle homogenates is not due to erythrocyte CA still present in the muscles after perfusion. For white skeletal muscles perfused in the same way as described here, this assumption has been confirmed by two other determinations: measurement of haemoglobin and CA isoenzyme pattern (Siffert & Gros, 1982). In the present study the determination of ^{51}Cr and ^{14}C radioactivities in homogenates indicates that only < 0.1 % (w/w) of erythrocytes were present in the perfused pooled skeletal muscles as well as in most of the perfused individual muscles and < 0.3 % in perfused heart and red thigh muscles, vastus intermedius and semimembranosus proprius. Table 2 shows that in the white skeletal muscles of the rabbit at most 2 % of the total CA activity found in the supernatants should be due to residual erythrocytes, whereas in heart and soleus muscle supernatants no significant activity in addition to that expected from erythrocyte contamination was detectable.

In the case of the particulate fractions of heart and skeletal muscle any contamination with soluble CAs is greatly decreased by the several washing steps performed with the homogenate pellets, so the high CA activity found in the Triton extract of the various particulate fractions is certainly not explicable by erythrocyte CA contamination. This is confirmed by the finding that the K_i of the CA present in these extracts differs by a factor of 5 from that of erythrocyte CA II.

It may be noted that removal of erythrocytes is only crucial for determination of sulphonamide-sensitive CA since measurements of CA III were done in the presence of 10 μM-acetazolamide, a concentration that fully inhibits erythrocyte CA isoenzymes.

CA III in homogenate supernatant

The present data indicate that the concentration of CA III in skeletal muscles is roughly correlated with their concentration of myoglobin (Table 3), the most notable exception being masseter which has a relatively high concentration of myoglobin but no detectable CA III activity. From a comparison of the CA III activities of the various individual muscles with their fibre composition, it appears likely that CA III is confined to slow twitch oxidative (SO) fibres. Only in muscles containing a high percentage of SO fibres, such as semimembranosus proprius and soleus (90 % SO; Lobley *et al.*, 1977), high activities of CA III were found, whereas in muscles containing mostly fast-twitch fibres, i.e. fast-twitch glycolytic (FG) and or fast-twitch oxidative-glycolytic (FOG) fibres, CA III activity is very low. The latter holds for muscles with a high percentage of FG fibres such as psoas (83 % FG; Lobley *et al.*, 1977) as well as for those with a relatively high content of FOG fibres such as tibialis anterior (41 % FOG, 54 % FG) and extensor digitorum longus (47 % FOG, 49 % FG; Lobley *et al.*, 1977). This assumption is in good agreement with histochemical results showing that CA III of skeletal muscle is only present in the cytoplasm of SO fibres (Shima *et al.*, 1983; Jeffery *et al.*, 1986; Väänänen *et al.*, 1986). That CA III is not present in rabbit heart has previously also been demonstrated by Carter *et al.* (1982).

The kinetic and inhibition constants given in Table 1 for rabbit CA III deserve a short comment. K_m for the CO_2 hydration reaction of CA III is in the same range as that for CA II. This is in contrast with an earlier report for rabbit CA III by Pullan & Noltman (1984), who found K_m values as high as 95 mM. It is also in contrast with some very high K_m values reported for the CA III of cat and pig muscle (Sanyal *et al.*, 1982; Pullan & Noltmann, 1984), but it is in the same range as values published for ox muscle CA III (9 mM; Engberg *et al.*, 1985) and for rat liver CA III (5 mM; Sanyal, 1984). This implies that the K_m is not very much higher than the concentrations of CO_2 occurring physiologically in muscle. The turnover number $k_{cat.}$, 35 s^{-1}, is considerably lower than other reported values, which at 25 °C range between 2000 and 4000 s^{-1}. This is due to the inhibitory effect of Cl^- on the activity of CA III (Sanyal *et al.*, 1982). The K_i for ethoxzolamide towards rabbit CA III given in Table 1 is in excellent agreement with the value obtained by Sanyal *et al.* (1982).

CA II in homogenate supernatants

In individual fast skeletal muscles of the rabbit (lines 4–8 of Table 2) as well as in pooled white muscles of the rabbit a sulphonamide-sensitive CA has been found in the cytosol. This isoenzyme has about the same K_i towards ethoxzolamide as CA of rabbit erythrocytes (0.7 nM; Siffert & Gros, 1982), which, as mentioned above, under the experimental conditions employed exhibit CA II activity only. Similarly, the K_i values towards acetazolamide for gastrocnemius and pooled white muscle supernatant, 28 nM and 20 nM, are nearly identical with those for

rabbit erythrocytes and for pure bovine CA II (17 nM; Bruns *et al.*, 1986). Indeed, affinity chromatography studies of Siffert & Gros (1982) indicated that the sulphonamide-sensitive CA in homogenate supernatants of pooled white skeletal muscles of the rabbit is CA II, and that the other cytosolic sulphonamide-sensitive isoenzyme, CA I, is absent from white muscle supernatants. Thus there is considerable evidence to show that the sulphonamide-sensitive CA found in fast rabbit muscles represents CA II.

In contrast with the results obtained with homogenate supernatants of pooled red rabbit muscles (Fig. 1, Table 1), no or only small activities of acetazolamide-sensitive CA were detectable in rabbit soleus and semimembranosus proprius/vastus intermedius. Since these muscles consist mostly of SO fibres, this suggests that SO fibres as well as heart muscle fibres contain no CA II in their cytosolic fraction. In the rat, CA II appears to be virtually absent from all fast as well as slow muscles studied (Table 2).

Considering the various percentages of FG and FOG fibre types (Lobley *et al.*, 1977) in the individual fast rabbit muscles in Table 2, it appears likely that FG as well as FOG fibres of this species contain CA II. If this reasoning is correct, it is not surprising that in pooled red muscles a cytosolic CA II is present in addition to CA III, whereas in the almost purely SO-type muscle, soleus, CA II is not detectable. Since SO as well as FOG fibres possess high levels of myoglobin (Holloszy & Booth, 1976), pooling of all muscles of reddish appearance is expected to yield cytosolic CA II originating from FOG as well as cytosolic CA III derived from SO fibres.

In contrast with our conclusion that CA II is absent from rat skeletal muscle, histochemical studies have demonstrated a sulphonamide-sensitive CA in the cytoplasm of FOG fibres of the rat (Riley *et al.*, 1982). With immunocytochemical methods, however, Jeffery *et al.* (1986) found a reactivity against CA II antisera not in FOG fibres but only in type I (SO) fibres in the form of spots; in addition, they found with CA I antisera a staining in the form of spots and staining of the sarcolemma in type I and IIA (SO and FOG) fibres. We do not know if there is a cross-reactivity between these antisera and the membrane-bound CA. Nevertheless, their staining pattern suggests that it may be the CA bound to the sarcoplasmic reticulum and sarcolemma (see below) which reacts with anti-(CA I) and anti-(CA II). In the cytoplasm of the heart no sulphonamide-sensitive CA has been observed histochemically (Carter *et al.*, 1982) or biochemically (Moynihan, 1977).

Sulphonamide-sensitive CA in the particulate fraction

The washed pellets from all rabbit muscles studied (Table 4) released, on treatment with detergent, a CA with hydratase activity that could be completely suppressed by 10 μM-acetazolamide. The K_i values with respect to acetazolamide obtained for this enzyme show, however, that it is different from cytosolic CA II. The K_i values, 73–94 nM, are three to six times higher than those for bovine CA II, rabbit erythrocyte CA II and cytosolic CA II of fast rabbit muscles (17–28 nM; Bruns *et al.*, 1986; and above). On the other hand, they are two to three times lower than the K_i value (200 nM) reported by Sanyal *et al.* (1981) for cytosolic CA I. The same observation of a K_i value intermediate between that for CA I and that for CA II has been made for the membrane-bound CA of kidney (Sanyal *et al.*, 1981) and lung (Whitney & Briggle, 1982).

Possible sources of the CA of the Triton extract are sarcoplasmic reticulum and sarcolemma. In the case of skeletal muscles, there is evidence for both localizations. Sarcoplasmic reticulum of red as well as white muscles contains CA (Bruns *et al.*, 1986), the K_i being in the same range as in our preparation. It is very likely therefore that sarcoplasmic reticulum contributes to the CA activity of the present membrane fraction. Direct evidence for CA associated with the sarcolemma of white as well as red muscles is provided by studies of isolated sarcolemmal vesicles of the rabbit (Wetzel & Gros, 1990). In the case of heart muscle the activity of the membrane-bound CA is three to eight times higher than in skeletal muscle, but the origin of this membrane-bound CA is not yet clear. In histochemical studies (our own unpublished observations), a pattern similar to that of skeletal muscle was observed: stained 'spots' within heart muscle cells and sarcolemmal staining indicating that there may also be CA associated with the sarcoplasmic reticulum and the sarcolemma. On the other hand, results from indicator-dilution studies (Zborowska-Sluis *et al.*, 1975), in which no change in the distribution space of $H^{14}CO_3^-$ was observed on addition of CA inhibitors to the perfusate, seem to suggest that no sarcolemmal CA is present in the heart. From surface pH measurements, de Hemptinne *et al.* (1987), however, concluded that an extracellular CA associated with heart sarcolemma exists. It appears possible therefore that heart, like skeletal muscle, possesses a membrane-

Table 4. CA activity in the particulate fraction of various striated rabbit muscles

CA activity in 1.2% (w/w) Triton X-100 extracts of pellets from muscle homogenates. The pellets were freed of cytosolic CA by several washing steps. The remaining contamination with cytosolic enzyme was estimated from the CA activity in the last supernatant. The inhibition constants towards acetazolamide (K_i) were determined by Maren's micromethod. All CA activities were completely suppressed by 10 μM-acetazolamide. Parameters are given as means ± S.D. (n is the number of animals whose muscles were studied; $n = 1$ if not otherwise stated).

Muscle	CA activity in Triton extract of homogenate pellet (units)	CA activity in supernatant (last washing) (units)	Specific activity of Triton extract (units/ml per mg of protein)	K_i (nM)
Heart ($n = 4$)	152 ± 37	1.2 ± 0.3	17.5 ± 1.3	94
Soleus ($n = 2$)	39	< 1	2.1	73
Vastus intermedius and semimembranosus proprius	60	< 1	2.5	–
Tibialis anterior	62	< 1	2.8	–
Extensor digitorum longus	61	< 1	–	–
Gastrocnemius (white)	60	< 1	4.1	–
Gastrocnemius intermedius	60	< 1	3.1	–
Masseter	115	< 1	6.7	83
Gracilis	52	< 1	5.5	–

bound CA in the sarcoplasmic reticulum as well as in the sarcolemma. Muscle mitochondria can be excluded as a source of the present Triton-extractable CA since (1) Bruns *et al.* (1986) have shown that rabbit muscle mitochondria have very little or no CA activity and (2) mitochondria are expected to be destroyed by the freezing, pulverizing and thawing process; thus any mitochondrial CA would be found in the cytosolic fraction.

Among the pellet extracts from all striated muscles studied, the highest CA activity was found in the extracts prepared from perfused hearts (Table 4). Nevertheless, the contribution of the present Triton-extractable CA to an overall CA activity in the initial homogenate is rather low. A rough estimate indicates that the CA activity measured in the Triton extract must be divided by 5–10 to give this overall activity, i.e. undiluted heart homogenate is expected to possess a membrane-bound CA activity of 15–30 units. This may explain why Moynihan (1977) found no significant CA activity in rat hearts homogenized in the presence of Triton, since he used unperfused hearts. On the basis of Moynihan's (1977) values for trapped blood, and expressed in the activity units as used in the present study, we estimate a CA activity in his heart homogenates due to trapped erythrocytes of 700–1000 units. Thus it appears impossible to detect an activity of 15–30 units.

Possible physiological roles of muscle CAs

For some years it has been known that the presence of CA in muscle is useful for the transfer of CO_2 from the site of production within muscle cells to the blood by means of two separate mechanisms: (1) facilitation of CO_2 diffusion within the muscle cell which has been shown to be due to CA III in red skeletal muscle (Gros & Dodgson, 1988) but which also occurs in the CA III-free white skeletal muscles and in the heart of rabbits (Romanowski *et al.*, 1991); (2) CA activity available in the interstitial space of skeletal muscle, due to sarcolemmal CA, is thought to accelerate the uptake of CO_2 by the blood (Forster & Crandall, 1975; Crandall *et al.*, 1977; Hill *et al.*, 1977; Bidani *et al.*, 1978; Geers *et al.*, 1985).

Another role of muscle CAs is the participation of the extracellular sarcolemmal CA in extracellular buffering (de Hemptinne *et al.*, 1987): CO_2/HCO_3^- is the major extracellular or interstitial buffer, but without CA its buffering action would be slow, and only in the presence of an extracellular–interstitial CA may it be fast enough to protect the interstitial space from drastic acidification, for example when lactic acid leaves muscle cells at the onset of heavy exercise. Considering the high anaerobic capacity of fast muscles as opposed to slow muscles it seems to make sense that the specific CA activity in the Triton extracts is highest for the fast muscles (2.8–6.7 units/ml per mg) and lower for the slow skeletal muscles (2.1–2.5 units/ml per mg).

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