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CALCIUM SENSITIZING ACTION OF CARNOSINE AND OTHER ENDOGENOUS IMIDAZOLES IN CHEMICALLY SKINNED STRIATED MUSCLE

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SUMMARY

1. The imidazole-containing compounds carnosine and homocarnosine, endogenous to skeletal and cardiac muscle, have been tested for effect on the contractile behaviour of chemically skinned (saponin or Triton X-100) skeletal and cardiac muscle.

2. Carnosine, at millimolar concentrations which are near physiological for many skeletal fibres, and in a concentration-dependent fashion, shifts the curve relating $[Ca^{2+}]$ to steady-state tension to lower $[Ca^{2+}]$ in both skeletal (frog but not crab) and cardiac (rat) muscle preparations.

3. Of other imidazoles endogenous to heart, homocarnosine is somewhat more effective, while *N*-acetyl L-histidine is much less so.

4. The maximum level of Ca^{2+}-activated force is increased significantly by homocarnosine in cardiac trabeculae.

5. We propose that the cellular imidazoles related to carnosine are natural 'Ca^{2+} sensitizers' in striated muscle. Alterations in their levels as a result of disease or training, and between different fibre types, may contribute to differences in contractile performance of the intact tissues.

INTRODUCTION

Much information about the role of Ca^{2+} ions in the activation of muscle has been derived from studies on skinned fibres. In such experiments it is generally hoped that the conditions approximate to those in the muscle *in vivo*, or depart from them in some defined way. The relationship between $[Ca^{2+}]$ and force, and the influence of other ions and molecules upon it, are of central interest. Wendt & Stephenson (1983) reported that millimolar levels of caffeine increase the Ca^{2+} sensitivity of the contractile machinery. This action will augment the longer-known ability of caffeine to induce Ca^{2+} release from the sarcoplasmic reticulum (SR). Caffeine is a methylxanthine and includes an imidazole ring in its structure (Fig. 1). Previous

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work has shown that the lone-pair nitrogen in such a ring is the key feature that distinguished which of caffeine's chemical relatives are able to cause contracture in cardiac and skeletal muscle (Chapman & Miller, 1974; Miller & Thieleczek, 1977). We sought an explanation of the enhancing effect of caffeine on Ca^{2+} sensitivity by considering whether analogous compounds exist in muscle cytoplasm whose action caffeine could be mimicking.

Cellular imidazoles, such as the dipeptides carnosine (β-alanyl L-histidine), discovered in bovine muscle over 90 years ago (Gulewitsch & Amiradzibi, 1900) and anserine (β-alanyl-1-methyl L-histidine), first detected in the goose, *Anser* (Tolkachevskaya, 1929; Ackerman, Timpe & Poller, 1929) often occur at millimolar levels in a variety of cell types, including muscle (for review see Crush, 1970). Their action as pH buffers is widely considered to be their most important role (Davey, 1960), but they have been implicated in a variety of other cellular functions including myosin ATPase regulation (Avena & Bowen, 1969), divalent cation binding (Brown, 1981), neurotransmission (Rochel & Margolis, 1982), modulation of muscle phosphorylases (Johnson & Aldstadt, 1984) and Ca^{2+} uptake by the SR (Lopina & Boldyrev, 1975). However, there is frequent comment in the literature to the effect that the true role of these dipeptides is not established (see Severin, Val'fson, Grigorovich & Soloveva, 1963; Severin, 1976). The potential of these natural imidazoles for 'calcium sensitizing' action encouraged us to analyse them for effectiveness on the contractile behaviour of skinned fibres.

Our hypothesis was that endogenous imidazole-containing compounds like carnosine might have a similar action to imidazole drugs, such as caffeine and sulmazole, and increase the apparent Ca^{2+} sensitivity of the contractile machinery.

Preliminary results have been reported to the Physiological Society (Harrison, Lamont & Miller, 1985).

METHODS

Cardiac muscle. Experiments were carried out on small, free-running trabeculae isolated from the right ventricle of the rat. The majority of preparations are taken from the base of the ventricle near the valves. The animals were killed by a blow to the head, the heart was then rapidly excised and flushed with a saline solution (Solution 'A' Table 1) at room temperature. A suitable trabecula (2–3 mm long and 80–180 μm in diameter) was excised and mounted for isometric force measurement.

Skeletal muscle. Some experiments were made on single fibres or small fibre bundles isolated from frog sartorius muscle (*Rana temporaria*), semi-membranosus (*Xenopus laevis*), cod fast or slow fibres (for methods see Altringham & Johnston, 1985) and crab (*Carcinus maenas*) walking-leg muscle fibres. These fibres were dissected under light liquid paraffin prior to chemical skinning.

Full details of the experimental system for mounting the preparations, detecting and recording isometric force, solution exchange, measuring sarcomere length (SL) and preparation dimensions, and solution chemistry have been published (Harrison, Lamont & Miller, 1988). In summary, for all types of preparation the muscle was snared at both ends with nylon monofilaments (25 μm diameter) emerging from stainless steel tubes (100 μm i.d., 200 μm o.d.) to give a final preparation length of 1–2 mm. The experimental solutions were carried in a series of wells (4·5 ml) in a Perspex block which was raised and lowered by two stepper motors operating under microprocessor control to effect the solution change. A modified Vickers M-17 microscope using the differential interference contrast system was used to measure sarcomere length. The microscope stage had been replaced with a small chamber to permit observation and adjustment of SL in preparations mounted for isometric tension recording. Sarcomere length was set and could be checked after the preparation was transferred to the solution change system. Except where stated otherwise, SL was set at 2·0–2·2 μm (cardiac muscle) and 2·2–2·3 μm (skeletal muscle). The rationale for the solution

composition, the method of calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, and the precautions for EGTA purity and the measurement of pH (expressed as pH_a to distinguish ion activity from concentration) are described in detail elsewhere (Miller & Smith, 1984, 1985; Smith & Miller, 1985; Harrison *et al.* 1988). The free Ca^{2+} concentration ($[Ca^{2+}]$ or expressed as $-\log[Ca^{2+}]$, pCa_c) was established in the first instance by

TABLE 1. Composition of solutions (all concentrations in mM)

Solution	A	B	C	D	E
NaCl	140	—	—	—	—
KCl	5·0	100	100	100	100
$MgCl_2$	1·0	7·0	7·0	7·0	7·0
CaEGTA	—	—	10	—	10
K_2EGTA	—	10·0	—	0·2	—
K_2HDTA	—	—	—	9·8	—
Na_2ATP	—	5·0	5·0	5·0	5·0
Na_2creatine phosphate	—	15	15	15	15
*HEPES	5·0	25	25	25	25
†KOH	—	15	15	15	15
NaOH	1·0	—	—	—	—
$CaCl_2$	2·0	—	—	—	0·1

B, C, D and E contain 30–50 IU per ml creatine phosphokinase. All solutions include 5 mM-D-glucose. HDTA, 1,6,diaminohexane-*N,N,N′,N′*-tetraacetic acid.

* The concentration of HEPES was altered where carnosine and imidazole were employed. See text for details.

† The amount of KOH was varied (10–20 mM) to adjust pH_a (see text above) to 7·0 in B, C, D and E.

Temperature 20–22 °C.

mixing solutions B and C (Table 1) in the desired ratios. Experiments were carried out at room temperature (20–22 °C). Checks on commercial supplies of carnosine and other compounds used confirmed that Ca^{2+} contamination was negligible.

Chemical skinning procedure. The mounted muscle was initially exposed to a 'relaxing' solution (B, Table 1) including either the cholesterol-precipitating agent saponin (50 μg/ml) or Triton X-100 (1 % v/v) for 20–30 min. The chemical skinning agent was then removed by washing in solution B. $[Ca^{2+}]$ was altered by switching the muscle from one bath to another.

Data handling. The tension signal was routinely digitized (12 bit) at appropriate rates. To avoid aliasing errors the signal was prefiltered with an active filter. A continuous chart recording (Linseis 1800) was also made. Tension responses are shown calibrated in either or both absolute force and relative force. For the latter, tension was normalized to maximum Ca^{2+}-activated force (C_{max}) which was determined at frequent intervals throughout the experiment. Provided that relatively short intervals elapsed between normalizing maxima, a linear interpolation between these test levels were found to describe adequately the decline of tension (see Harrison *et al.* 1988 for details). The relationship between $[Ca^{2+}]$ and tension was satisfactorily described by eqn (1), the Hill equation, with values for K_{app} and 'h' appropriate to that preparation (see Harrison *et al.* 1988 for details). K_{app} has the units M^{-h}. $K_{\frac{1}{2}}$, the reciprocal of the $[Ca^{2+}]$ required for half-maximum activation, is defined as the hth root of K_{app}.

$$\frac{C}{C_{max}} = \frac{K_{app}[Ca^{2+}]^h}{(1+K_{app}[Ca^{2+}]^h)}. \tag{1}$$

RESULTS

Effects on the Ca^{2+} sensitivity of contractile proteins

The chemical relationship of the imidazole-containing compound caffeine with dipeptides such as carnosine and the benzimidazole drug sulmazole is illustrated in Fig. 1. Extrapolating from the results of Chapman & Miller (1974), we hypothesized

that the imidazole ring might be sufficient to augment the myofibrillar calcium sensitivity in caffeine-like manner. This idea was assessed by applying the imidazoles to chemically skinned striated muscle preparations. Preparations were subjected to chemical skinning techniques which are 'selective' (using saponin, which leaves the

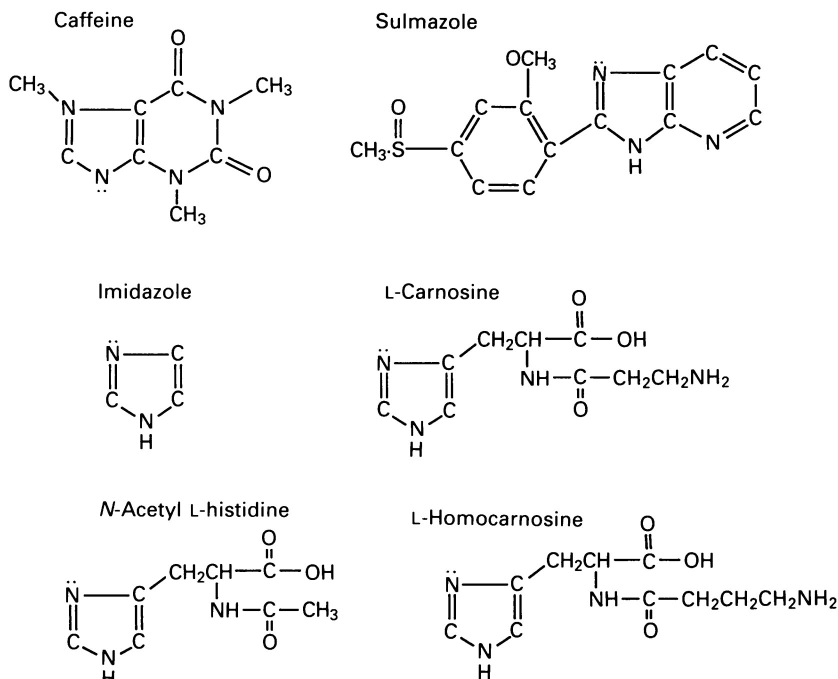

Fig. 1. The chemical structures of imidazole, sulmazole, caffeine, carnosine (β-alanyl L-histidine), homocarnosine (γ-amino butyryl L-histidine) and *N*-acetyl L-histidine.

intracellular membranes functionally intact and retains many cytoplasmic enzymes; Endo & Kitazawa, 1978) or complete (using Triton X-100 which destroys all membranes; Helcius & Simon, 1975). In a series of experiments, points on the curve relating $[Ca^{2+}]$ to tension were determined in the presence and absence of caffeine, L-carnosine, imidazole, *N*-acetyl L-histidine, homocarnosine, anserine or sulmazole (AR-L 115BS) at a series of concentrations.

Figure 2*A* shows a trace obtained with a frog sartorius fibre chemically skinned with Triton X-100 to ensure that all cellular membranes had been destroyed. The preparation was half-maximally activated by increasing $[Ca^{2+}]$ from a sub-threshold level. The solution included 15 mM-carnosine, the physiological concentration in this muscle (Burton, 1983). We take the curve determined in the absence of imidazoles (in this case with 40 mM-HEPES as pH buffer) as the standard one. HEPES was replaced in the solutions (B and C, Table 1) on a mole for mole basis by the test substance so that the ionic strength (*I*) and total pH buffer capacity remained the same within a few millimolar (carnosine's pK_{b2} for example is 6·75, 25 °C, $I = 0·1$ M). Checks with an additional 5 mM-KCl show that modest alterations in $[K^+]$ and ionic strength under the conditions prevailing in our solutions had no significant effect.

Once a steady state was achieved, the carnosine was removed and then reapplied. Tension fell when carnosine was removed, and increased when it was reapplied, at a rate consistent with diffusion, demonstrating the reversibility of carnosine's action. The alteration in tension indicates an enhancement of apparent Ca^{2+} sensitivity by

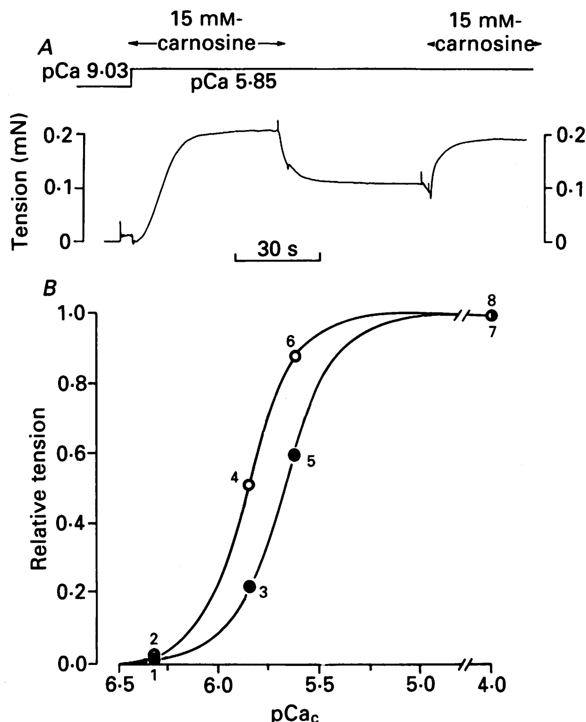

Fig. 2. Increase in Ca^{2+} sensitivity in skeletal muscle by carnosine. Panel *A* shows the force produced by a small bundle of Triton-treated frog (*Rana temporaria*) fibres. The effect of removing and reapplying 15 mM-carnosine at constant $[Ca^{2+}]$ is shown. The pCa_c (see Methods section) chosen (5·85) evoked about half-maximal activation in this preparation. The solutions correspond to those in Table 1 except K^+ 80 mM, Cl^- 74 mM, HEPES plus carnosine 40 mM, ionic strength 0·14 M. Panel *B* shows the pCa–tension relationship from a single frog sartorius fibre. Results in the absence (●), and presence (○) of 15 mM-carnosine. pCas were recalculated for the slightly different ionic conditions prevailing in these solutions from those in Table 1. The force determinations were made from the relaxed state in the order indicated by the small numbers near each point. $\log K_{\frac{1}{2}}$ increased from 5·65 to 5·85 and h from 3·3 to 3·8 under carnosine.

carnosine: the dipeptide in this respect behaves very like caffeine, as our hypothesis had predicted on the basis of the chemical structure. A few points on the curve relating pCa to tension were established in four skeletal fibre preparations. An example is shown in Fig. 2*B*. The shift in Ca^{2+} sensitivity (only tentatively established with these observations) produced by 15 mM-carnosine (expressed as the reduction in $\log K_{\frac{1}{2}}$, the $[Ca^{2+}]$ necessary for half-maximum activation), was 0·19 (range 0·10 to 0·38) units. C_{max} fell much more rapidly in these skeletal muscle preparations than in heart, restricting the number of observations that could be made.

Many invertebrate muscles have very low levels of histidine dipeptides. Parker & Ring (1970) have shown their ATPases to be unaffected by imidazole dipeptides. Therefore, the action of both carnosine and sulmazole was tested on fibres from crab (*Carcinus maenus*) walking leg (solutions as in Table 1 but 40 mM extra KCl added).

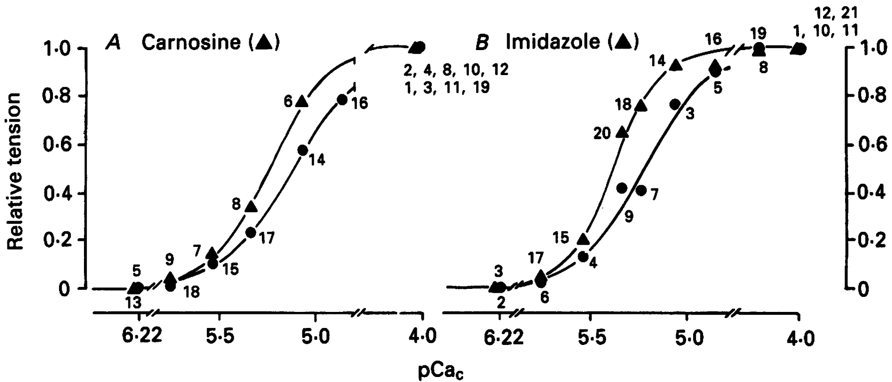

Fig. 3. Effects of carnosine (*A*, ▲) and imidazole (*B*, ▲), each at 10 mM, on the relationship between [Ca^{2+}] (expressed as pCa$_c$, see Methods) and steady-state isometric force (standard curves, ●). The preparations were from rat right ventricle, selectively skinned with saponin (see Methods). The small numbers indicate the sequence in which contractures were evoked. Forces are normalized to C_{max} under each test condition: in *A* carnosine potentiated C_{max} to 108% (mean), in *B* imidazole depressed C_{max} to 78%.

Carnosine (25 mM) and sulmazole (10 mM) were both ineffective in altering either Ca^{2+} sensitivity or maximum Ca^{2+}-activated force (three preparations). The negative result with sulmazole confirms earlier reports by Ashley & Griffiths (1985) for crustacean (*Balanus*) muscle.

Preliminary experiments with Triton-treated red (slow) and white (fast) fibres from the cod show that carnosine and sulmazole increases Ca^{2+} sensitivity in both types of fibre (C. Lamont. D. J. Miller & I. A. Johnston, unpublished observations).

Figure 3 shows examples of the increase in Ca^{2+} sensitivity produced by 10 mM-L-carnosine (Fig. 3*A*) and imidazole (Fig. 3*B*), each in a rat ventricular trabecula treated with saponin. The points are determined as steady-state force achieved at a given [Ca^{2+}]. In this example, each test was made by switching directly from solution D (0·2 mM-EGTA$_{total}$, Table 1) to the activating solution at the desired [Ca^{2+}] (10 mM-EGTA$_{total}$). The muscle was then relaxed with solution B. In some experiments, the contractures were evolved at the test [Ca^{2+}] and then the test compound was applied. In other experiments two or more test [Ca^{2+}]s were applied sequentially either with or without the test compound present. Such runs were made repeatedly to check for time or sequence dependence of imidazole action: none was found. Checks were made in several experiments that pCa 4 (100 μM-Ca^{2+}) reliably evokes maximum force. At the SL used in these experiments peak force is constant between about pCa 4·2 and 3·5 ($6·3 \times 10^{-5}$–$3·2 \times 10^{-4}$ M-Ca^{2+}, see for example Fig. 3*B*, Fig. 5). For the example in Fig. 3, $\log K_{\frac{1}{2}}$ (see eqn (1) and text) was increased from 5·10 to 5·23 (*A*) and 5·21 to 5·37 (*B*), and the 'Hill' exponent ('*h*') from 2·29 to 2·81 (*A*) and 2·62 to 3·58 (*B*). The shift produced by carnosine is similar to that reported

for 10 mM-caffeine (Wendt & Stephenson, 1983), which we have confirmed (data not shown). Imidazole itself was approximately equipotent to carnosine, although C_{max} was depressed (see figure legend and later). In five experiments with 10 mM-carnosine, the shift (increase) in $\log K_{\frac{1}{2}}$ was 0·074 ± 0·030 (mean ± S.E.M.). We also find

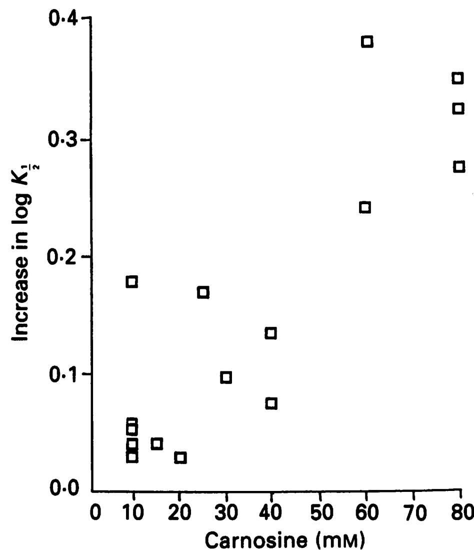

Fig. 4. Dose–response curve of carnosine on rat ventricle muscle. The extent of the increase in $\log K_{\frac{1}{2}}$ ($\log K_{\frac{1}{2}}$ carnosine − $\log K_{\frac{1}{2}}$ control) is plotted on the ordinate against the carnosine concentration (abscissa).

that sulmazole, a benzimidazole cardiotonic drug (Herzig, Feile & Rüegg, 1981), increases Ca^{2+} sensitivity, as reported by others. We have found that, at millimolar concentration, sulmazole continues to work in a dose-dependent manner, producing much greater sensitizing effect than caffeine or carnosine (e.g. 0·32 log unit at 10 mM, not illustrated).

The Ca^{2+} sensitivity is not different in preparations treated with either saponin or Triton X-100, as we have reported previously (Miller & Smith, 1985). Carnosine was effective in preparations treated with either agent, suggesting that the cellular constituents retained in the saponin-treated state, but lost after Triton X-100 treatment, are not required for carnosine's Ca^{2+} sensitizing action.

The steepening of the pCa–tension curve seen in these examples is not always found. In a series of sixteen pCa–tension curves obtained in cardiac muscle with various concentrations of carnosine, the value of the Hill coefficient, h, increased in four remained unaltered in two and fell in ten cases. (An example of a shallower curve is described later, Fig. 5.)

The concentration dependence of carnosine's Ca^{2+} sensitizing action has not been studied in detail. However, Fig. 4 shows pooled data for the shift in $\log K_{\frac{1}{2}}$ at various carnosine concentrations up to 80 mM in rat cardiac muscle. The effect does not appear to saturate, although complications associated with the change in ionic strength cannot be satisfactorily compensated for in experiments of this kind.

(Increased ionic strength tends to depress peak force, e.g. Ashley & Moisescu, 1977; Kentish, 1984; with the additional effect that increased monovalent cation concentration (K^+, Na^+) depresses Ca^{2+} sensitivity; Fink, Stephenson & Williams, 1986.)

N-acetyl L-histidine (Fig. 1), another imidazole found in heart muscle from the frog (Kuroda & Ikoma, 1966) and mammals (Sobue, Konishi & Nakajima, 1975; Crichton, Lamont, Miller & O'Dowd, 1988; O'Dowd, Robins & Miller, 1988), was tested in several cardiac preparations. It has a much lower Ca^{2+} sensitizing effect, producing no clear alteration in $\log K_{\frac{1}{2}}$. However, at lower degrees of activation, force is generally increased very slightly upon addition of *N*-acetyl L-histidine. Checks of the kind described earlier confirm that the small differences in $[K^+]$ or ionic strength between solutions with or without *N*-acetyl L-histidine (at 10 mM) are insufficient to explain any differences observed. (Note, *N*-acetyl L-histidine is acidic while carnosine is basic.)

Work from our laboratory has reported the existence of homocarnosine at millimolar concentration in rat heart muscle. Of several endogenous imidazoles identified, homocarnosine is the most prevalent in ventricle (House, Miller & O'Dowd, 1989*a*, *b*). Since homocarnosine is available commercially in pure form, we have tested its actions in the same way as carnosine. Figure 5*A* shows an example of the effect of 10 mM-homocarnosine on a rat ventricle trabecula. In five preparations, the shift (increase) in $\log K_{\frac{1}{2}}$ was $0{\cdot}127 \pm 0{\cdot}009$ (mean ± S.E.M.).

A cardiac preparation was tested with anserine. A sensitizing effect, lower than that for homocarnosine was found, as illustrated in the example in Fig. 5*B* (see figure legend for details). (Anserine is only available commercially as the nitrate salt. Control solutions included 10 mM-KNO_3.)

The effect on maximum calcium-activated tension

Forces are normalized to maximum Ca^{2+}-activated contracture tension (C_{max}) under each condition for the results presented so far. While this normalization is necessary to reveal the nature of the shifts in steepness and $K_{\frac{1}{2}}$, it obscures a second feature of the action of these compounds on absolute force levels. The myoplasmic imidazoles increase maximum Ca^{2+}-activated force slightly in the same concentration range as they affect Ca^{2+} sensitivity. In cardiac muscle, homocarnosine (10 mM) increased C_{max} by $16{\cdot}5 \pm 1{\cdot}9$ % (S.E.M., $n = 5$ preparations), and carnosine (10 mM) only minimally (by $1{\cdot}4 \pm 1{\cdot}6$ %, $n = 5$ preparations). The problems associated with ionic strength (or $[K^+]$ changes noted above) also apply when measuring peak force; increasing ionic strength decreases C_{max} (e.g. Kentish, 1984). Thus the effects of higher concentrations of carnosine cannot be determined straightforwardly. Sulmazole, which is a more potent Ca^{2+} sensitizer than carnosine, also increases C_{max} substantially, in one example by 50 % at 10 mM. Caffeine had little effect on peak force (peak force $101{\cdot}5 \pm 2{\cdot}24$ of C_{max} at 10–20 mM, several observations in each of four preparations) in contrast to the slight reduction reported by Wendt & Stephenson (1983). Imidazole (10–20 mM) consistently reduced C_{max} (to 80–94 % range for three preparations); a greater reduction was seen (in two preparations) with 60 mM-imidazole but the associated ionic strength increase was not fully compensated and will have contributed to the effect (e.g. Ashley & Moisescu, 1977). We have not

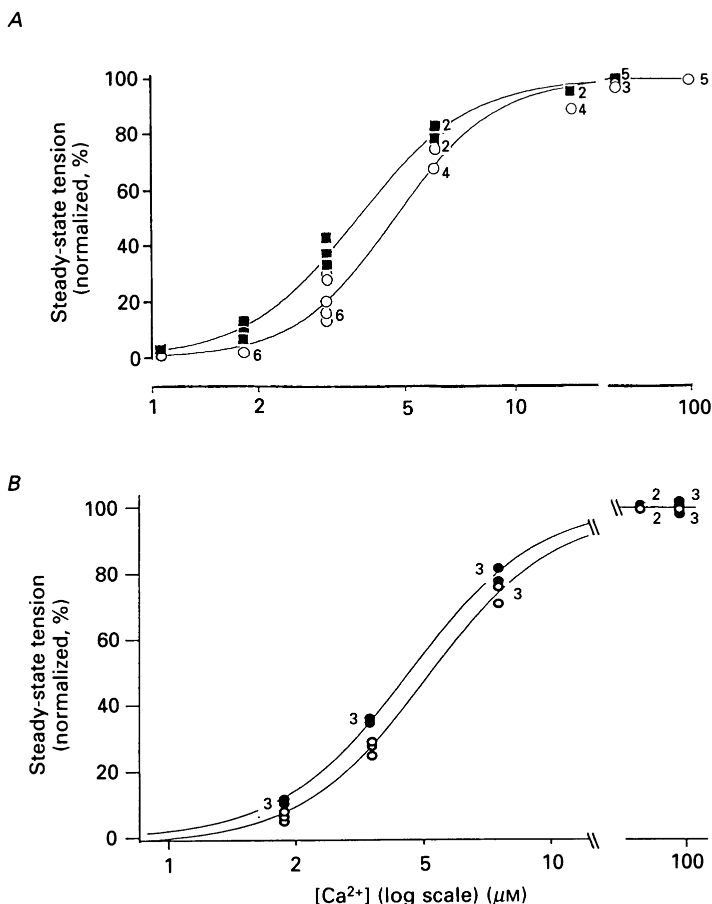

Fig. 5. Effects of homocarnosine or anserine on Ca^{2+} sensitivity of force production in a rat cardiac trabecula (○, control solution; closed symbols, plus 10 mM test compound). The panels show examples of the shift in Ca^{2+} sensitivity (forces normalized) produced in experiments of this kind. Small numbers indicate how many observations the symbol represents. Panel *A*, effects of 10 mM-homocarnosine. In the experiment illustrated, C_{max} was enhanced to $118{\cdot}0 \pm 1{\cdot}3$ % (mean ± S.E.M.). $\log K_{\frac{1}{2}}$ for the muscle increased from 5·34 ($h = 2{\cdot}81$) to 5·44 ($h = 2{\cdot}86$). Panel *B*, effects of 10 mM-anserine (as the nitrate salt: control solutions also included 10 mM-KNO_3). $\log K_{\frac{1}{2}}$ for the muscle increased from 5·37 ($h = 2{\cdot}93$) to 5·42 ($h = 2{\cdot}89$) in the presence of anserine. Maximum Ca^{2+}-activated force was not significantly altered by anserine ($100{\cdot}7 \pm 1{\cdot}1$ %).

studied the effect of these compounds on peak force in skeletal fibres, although this could be of obvious interest for physiological or pathological performance.

DISCUSSION

Ca^{2+} sensitizing action of endogenous and artificial imidazoles

We investigated the natural imidazoles of muscle because they have a certain chemical similarity to methylxanthines such as caffeine. Until the report by Wendt & Stephenson (1983), it had been tacitly assumed by most workers that caffeine had

no effect on the Ca^{2+} sensitivity of the contractile proteins. This view followed reports from Korey (1950), Hasselbach (1953) and Hasselbach & Weber (1955) that caffeine was without effect on the superprecipitation reaction of extracted actomyosin systems. At least one of the earlier reports (Huddart, 1971) supporting this idea was obtained with invertebrate skeletal fibres. We have now found that both carnosine and the caffeine-like benzimidazole drug sulmazole have no effect on crab fibres which might help to explain the apparent contradiction. This observation is consistent with the ineffectiveness of sulmazole reported by Ashley & Griffiths (1985) for another crustacean, the barnacle, but not with their equally negative findings for frog.

As noted by others (e.g. Wendt & Stephenson, 1983) it is important to determine sensitivity shifts on individual preparations; variations in absolute Ca^{2+} sensitivity between individual muscles tend to obscure these effects if all data are pooled. This feature may help to explain why others may have overlooked the Ca^{2+} sensitizing action of imidazole in particular. A change in the shape of the pCa–tension relationship is potentially important in muscle that is less than fully activated as is the case for the heart (Fabiato, 1981). A shallower relationship means that force is increased disproportionately when activation is low. This feature has been reported for Ca^{2+} sensitizing drugs such as sulmazole, pimobendan and ORG30029 (Miller & Steele, 1990); it may be shared by carnosine and homocarnosine although our data do not permit a definitive conclusion. Alteration in Ca^{2+} sensitivity and the shape of the $[Ca^{2+}]$–force relationship are less significant for skeletal fibres which are usually assumed to be near-maximally activated. However, Ca^{2+} sensitizing would accelerate the onset of force development and possibly delay relaxation.

The Ca^{2+} sensitizing effects we report above develop after selective (saponin) or complete (Triton X-100) skinning of the muscle which suggests that no cytoplasmic or membrane-bound enzyme systems intermediate in the actions of the imidazoles; the same was concluded in the case of caffeine (Wendt & Stephenson, 1983). Changes in pH buffering capacity, ionic strength or Ca^{2+} contamination have been allowed for, compensated or otherwise ruled out as possible artifactual explanations for the effects observed. We have checked that for caffeine, at least, the ability to Ca^{2+}-sensitize is independent of SL over the range 1·8–2·1 μm. The experiments reported here on a variety of muscle types and species, as its general in the field, were done at 20 °C; it is possible that the effects described could be significantly influenced by temperature.

Imidazole is included by many workers in the experimental media applied to intracellular systems; this will go some way to compensating for the absence of natural imidazoles although imidazole itself has inhibitory effects on maximum force production as reported in the Results section. (The use of imidazole as a pH buffer for intracellular systems was even more widespread until the introduction of the zwitterionic 'Good's' buffers reported by Good, Winget, Winter, Connolly, Izawa & Singh in 1966.) The sensitivity to sulmazole of dog cardiac myofibrillar ATPase and Ca^{2+} binding was reported by Solaro & Rüegg (1982). It is interesting to note that these authors used 20 mM-imidazole throughout their study since we find that this is a submaximal dose for increased Ca^{2+} sensitivity; the shifts they report for sulmazole are perhaps less than might have been observed had they used a non-imidazole pH

buffer. However, our observations with both carnosine and sulmazole, and those reported for other Ca^{2+} sensitizing drugs (e.g. Miller & Steele, 1990), do not suggest a narrow limit to the scope for increasing $K_{\frac{1}{2}}$ by Ca^{2+} sensitizing compounds (see for example Fig. 4).

Imidazole structure and activity

Clearly, there are substantial structural differences between the imidazole-containing compounds that we have tested (Fig. 1). We conclude tentatively that molecules with a 'lone-pair' nitrogen atom is an imidazole ring in their structure (Fig. 1) are able to increase myofibrillar Ca^{2+} sensitivity. However, the chemical requirements for producing Ca^{2+} sensitization are not yet fully understood.

Mode of action on the regulatory proteins

By analogy with the effects of Ca^{2+} sensitizers such as sulmazole and pimobendan, it seems likely that carnosine and its relatives might increase Ca^{2+} binding to troponin-C (Solaro & Rüegg, 1982; Jacquet & Heilmeyer, 1987). However, it has been reported that caffeine does not affect Ca^{2+} binding by isolated cardiac or skeletal troponin-C (Kentish & Palmer, 1989). This could imply a different mode of action (Steele, Smith & Miller, 1990). However, there could be significant differences in the behaviour of the regulatory proteins between the isolated state and in the complex physico-chemical environment of the myofilament lattice. In any case, functional experiments of the type reported here cannot give direct evidence about the nature of the chemical interactions involved.

Physiological and pathological implications

We have investigated the effects of millimolar concentrations of carnosine (and *N*-acetyl L-histidine) which fall inside the physiological range for skeletal muscle and some hearts. New evidence (see O'Dowd *et al.* 1988; House *et al.* 1989*a*, *b*) suggests that the total level of histidyl compounds of this type in mammalian heart may be several millimoles although carnosine and *N*-acetyl L-histidine only represent a small fraction of the total. Homocarnosine is a significant constituent of rat ventricle and its ability to Ca^{2+}-sensitize and promote maximum Ca^{2+}-activated force, together with the Ca^{2+} sensitizing effect of anserine, have been described here. The effects observed develop over the millimolar (physiological) concentration range implying that physiological or pathological alterations in the concentration of carnosine-like substances would alter Ca^{2+} sensitivity in muscle. Currently, a complete range of the relevant endogenous imidazoles is not available for us to investigate the effects of the normal cellular milieu. The possibility of significant interactions between the various compounds in their effect on muscle contraction thus cannot yet be excluded.

Since myofilament regulatory proteins seem to be affected by cellular imidazoles, it is possible that other Ca^{2+} sensitive systems in muscle and non-muscle cells might be similarly influenced.

These observations may be relevant in several areas of normal and pathological muscle physiology, of which the following three are examples. (1) Carnosine or homocarnosine increase Ca^{2+} sensitivity and peak force production. Concentrations

of carnosine (and anserine) differ widely amongst different muscle fibre types, carnosine generally being much higher in fast fibres. The difference in Ca^{2+} sensitivity found between mammalian fast and slow fibres in experiments with skinned fibres (Stephenson & Williams, 1982) might be reduced if physiological levels of the imidazoles were to be present in the bathing media. (2) Carnosine concentrations might be low in dystrophic muscle (Strepanova & Grinio, 1968), in denervated muscle (Johnson & Altstadt, 1984), and in skeletal muscle after trauma (Fisher, Amend, Strumeyer & Fisher, 1978) and they alter during development (Fisher, Amend & Strumeyer, 1977). (3) Carnosine has a beneficial effect when included in cardioplegic solutions (Gercken, Bischoff & Trotz, 1980) albeit ostensibly as a pH buffer. The alterations of muscle function in (2) and (3) may be understood, at least in part, in terms of the present findings. Carnosine deficit would reduce Ca^{2+} sensitivity and contribute to diminished muscle performance. How far these features can be attributed to the many endogenous imidazoles other than carnosine (or homocarnosine which was similar actions) that exist in muscle, particularly the heart (see O'Dowd *et al.* 1988), has yet to be established. The relatively low potency of *N*-acetyl L-histidine, and slightly greater potency of homocarnosine, than carnosine itself, shows that small structural differences in the imidazoles can modulate their actions considerably. Thus the variety of endogenous imidazoles rather than their total concentration may be more relevant physiologically, and hence pathologically.

The use of the direct methods for measuring intracellular [Ca^{2+}] during muscle activity has produced evidence that the free [Ca^{2+}] achieved during activity in the intact tissue may be significantly lower than that implied from work on skinned muscle fibres and isolated proteins (Yue, Marban & Wier, 1986). The present findings support the view that important Ca^{2+} sensitizing components of the sarcoplasm are lost in various disrupted cell systems since carnosine and other cellular imidazoles represent such components.

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