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ISOVALERYLCARNITINE IS A SPECIFIC ACTIVATOR OF THE HIGH CALCIUM REQUIRING CALPAIN FORMS

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Isovalerylcarnitine, a product of the catabolism of L-leucine, is a potent activator of rat calpains isolated from erythrocytes, kidney, liver, skeletal and heart muscle. Only calpains II, but not calpains I, are activated by IVC, with the only exception of rat erythrocyte calpain I, the only species present in these cells which has a Ca^{2+} requirement higher than that of most calpain I isoenzymes. Activation by IVC involves a dual effect: 1) a ten fold increase in the affinity of calpain for Ca^{2+}, and 2) an increase in the Vmax 1.3-1.6 fold above the values observed with the native enzymes at saturating [Ca^{2+}] as well as with the autolyzed fully active calpain form at 5 μM Ca^{2+}. The increased affinity for calcium results in an increased rate of autoproteolysis of calpain II. Activation by IVC is additive to that promoted by interaction (or association) to phospholipids vesicles.

Together these results suggest that IVC may operate as a selective activator of calpain both in the cytosol and at the membrane level; in the latter case in synergism with the activation induced by association of the proteinase to the cell membrane.

Most mammalian cells contain two distinct isoenzymes of calpain: calpain I requiring approximately 5-50 μM Ca^{2+} and calpain II 0.2-0.6 mM Ca^{2+} for half maximum activity (for reviews see Refs. 1-4). Exceptions are represented by calpains from human (1) and rat erythrocytes (5) and human neutrophils (6) which contain only calpain I isoform. In order to express the activity at the very low cytosolic Ca^{2+} concentration, calpains must increase their affinity for Ca^{2+}. This can be promoted by either: a) the presence of saturating amounts of digestible substrates (7), b) the association to the cell membranes (8-10) or to a natural

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cytoskeletal associated activator (11), c) interaction with isovalerylcarnitine (IVC) (12). By increasing their Ca^{2+} affinity, calpains can be autoproteolytically converted to a form requiring low concentrations of Ca^{2+} (1-4) or reacquire the native properties following release from its membrane bound form (1, 5, 6). Considering that IVC exhibits an activating effect on calpain from human neutrophils (12), in the present study we have examined the effect of this carnitine ester on both calpains I and II isolated from various rat tissues. Definitive evidences are provided that IVC exerts a potent activating effect which is specifically directed on calpains II, affecting both the V_{max} and the Ca^{2+} requirement of this class of proteinases.

MATERIAL AND METHODS

Purification of calpain isozymes. Calpain I and II were separated with the following procedure. Minced tissues were suspended in 5 vol (v/w) of 0.25 M sucrose containing 1 mM MSH and 1 mM EDTA and disrupted in a glass-Teflon Potter-Elvehjem homogenizer (3 strokes) followed by sonication (4 bursts of 10 sec each). The particulates were discarded by centrifugation at 100,000 xg for 10 min and the clear supernatant loaded on a DEAE DE 32 column, previously equilibrated with 50 mM sodium acetate, pH 6.7, containing 0.5 mM MSH and 1 mM EDTA. The column was washed with the same buffer until the optical density at 280 nm of the effluent was below 0.02 and the absorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.4 M. Calpain activity was eluted in two distinct peaks. The first one (eluted at 60-80 mM NaCl) contains Calpain I activity, the second one (eluted at 250-280 mM NaCl) contains calpain II activity. Calpastatin activity appears as a single peak in between the two of calpain activity at approximately 140-160 mM NaCl. These peaks of proteinase activity were separately collected concentrated by ultrafiltration to approximately 3-4 ml and dialyzed against 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA and 0.5 mM MSH. The subsequent steps were identical to those previously reported for the purification procedure (13, 14, 15). Membrane vesicles were prepared as reported in (16, 17).

Calpain activity was assayed using denatured globin as substrate as previously reported (6). One unit was defined as the amount that causes the release of 1 umol of free amino groups per minute in the specified conditions.

The acylcarnitine derivatives were provided by Sigma Tau, Pomezia, Italy.

RESULTS

Activation of different calpain isoforms by IVC. Calpains I isolated from rat kidney, liver, heart and skeletal muscle, which

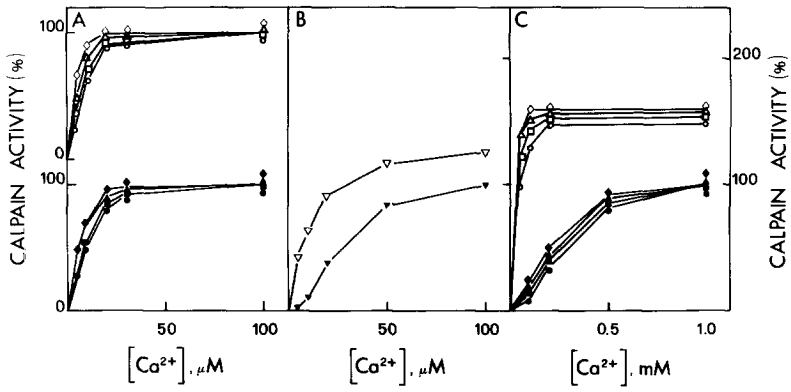

FIG. 1. (A) Effect of IVC on the Ca^{2+} requirement of calpain I isolated from various rat tissues. Calpain I was partially purified from rat kidney (◇), liver (△), skeletal muscle (□) and heart (○) as described in Methods. Calpain activity was assayed using human denatured globin as substrate with the indicated concentrations of Ca^{2+}, in the absence (closed symbols) or in the presence (open symbols) of 2 mM IVC.
(B) Effect of IVC on Ca^{2+} requirement of calpain I isolated from rat erythrocytes. Calpain activity was assayed as described in Methods with the indicated amounts of Ca^{2+} in the absence (▼) or in the presence (▽) of 2 mM IVC.
(C) Effect of IVC on the Ca^{2+} requirement of calpain II isolated from various rat tissues. Calpain II was purified from rat kidney (◇), liver (△), skeletal muscle (□) and heart (○) as described in Methods. Calpain activity was assayed using human denatured globin as substrate with the indicated amount of Ca^{2+} in the absence (closed symbols) or in the presence (open symbols) of 2 mM IVC.

express 1/2 V_{max} at approximately 5 μM Ca^{2+}, showed no change in the catalytic properties in response to IVC even at the highest concentrations used (Fig. 1A). Rat erythrocyte calpain, the only isoenzyme form present in these cells and not susceptible to autoproteolytic activation (5), was stimulated by IVC (Fig. 1B) with kinetic similar to that previously observed for the human neutrophil enzyme (12). On the contrary, addition of IVC promoted on all calpain II forms examined expression of full catalytic activity at low concentrations of Ca^{2+} and a 1.5 fold increase in the V_{max} at high concentrations of Ca^{2+} (Fig. 1C). This dual effect is dose dependent, 1/2 maximal and full activation being reached at IVC concentrations of about 0.5 and 1.0 mM respectively (data not shown). The D-isomer of IVC and the acylcarnitine derived from valine and isoleucine respectively showed a much lower activating

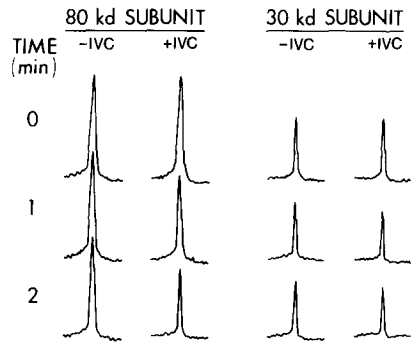

FIG. 2. Effect of IVC on the autolysis of the skeletal muscle calpain II. Purified calpain II was incubated for the indicated times with 0.1 mM Ca^{2+} in the absence or in the presence of 2 mM IVC. The samples were then submitted to SDS-PAGE. The stained gels were scanned with a Shimatzu LS-9000 densitometer.

effects (less than 25%) even at concentrations above 2 mM (data not shown).

Effect of IVC on calpains II autolytic activation. In order to investigate the activating mechanism of IVC, the time course of the autolysis of calpain II from rat skeletal muscle has been followed at 0.1 mM Ca^{2+} in the presence or absence of 2 mM IVC. The sequence of the results reported in Fig. 2 shows that IVC significantly accelerates the rate of autolysis of both 80 Kd and 30 Kd subunits (15). Similar results have been obtained with calpains II isolated from heart, kidney and liver (not shown). It would then appear that in calpains II both the increased sensitivity to Ca^{2+} and autolysis - consequent or concomitant events - are influenced by IVC. Furthermore, in the presence of both 20 μM Ca^{2+} and an excess of substrate the rate of autolytic activation of calpains II was enhanced two fold but 10 fold upon a further addition of 2 mM IVC (Fig. 3A). These data confirm previous observations (7, 18) and demonstrate that IVC increases the rate of the autolytic process even when Ca^{2+} requirements have been decreased by the presence of substrates. As shown in Fig. 3B the addition of IVC significantly increased the V_{max} of fully autoproteolytically acti-

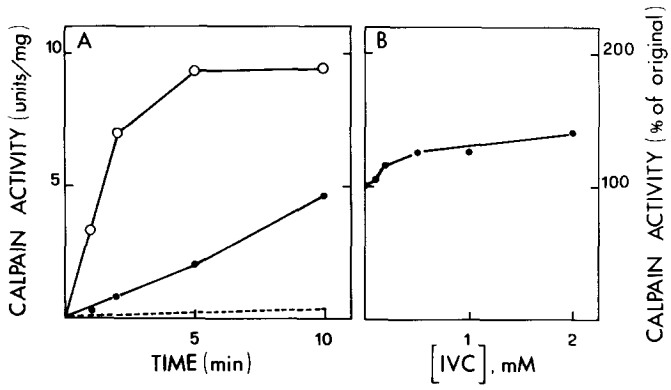

FIG. 3. (A) Effect of IVC on the rate of conversion of calpain II isolated from skeletal muscle. Calpain was incubated with 2 mg/ml substrate, 20 μM Ca^{2+} in the absence (●) or in the presence (○) of 2 mM IVC. At the time indicated, aliquots of the incubation mixtures were removed and assayed for calpain activity in 5 μM Ca^{2+}. The concentration of IVC in the assay mixtures was reduced to 0.05 mM, amount insufficient to produce any effects on the enzyme activity. The dotted lines refer to an incubation mixture in which the substrate and IVC were omitted.
(B) Effect of IVC on the catalytic activity of the autolyzed form of calpain II isolated from skeletal muscle. Native calpain II was incubated for 1 min with 0.1 mM Ca^{2+} and 2 mg/ml substrate. The autolysis was stopped by addition of EDTA, and calpain activity was assayed at 5 μM Ca^{2+} in the presence of the indicated amounts of IVC.

vated calpain II, in a concentration-dependent manner. Since the autolyzed calpains II represent the unmasked active form of the proteinase, the additional activation by IVC supports the assumption of conformational changes induced by this carnitine ester.

Synergistic activating effect of IVC and phospholipids. The results reported in Table I demonstrate a clear additive effect of IVC and phospholipid vesicles on the activity of all calpain forms tested but not, as expected, on the activity of typical calpains I. These results suggest that phospholipid and IVC binding to different sites of the sensitive calpains, synergistically increases their activity at μM Ca^{2+} a concentration at which otherwise these calpains are inactive. The possibility that binding to the membrane and interaction with IVC may represent cooperative mechanisms for the modulation of calpain activity during intracellular proteolysis is consistently supported by these results.

Table I

Source and type of Enzyme	Calpain activity (% of maximal in 1 mM Ca^{2+}) in the presence of					
	none	PL vesicles	IVC, 0.2 mM	PL vesicles +IVC, 0.2 mM	IVC 2 mM	PL vesicles +IVC, 2 mM
Skeletal muscle, Calpain I	18	34	19	32	18	36
Rat erythrocyte, Calpain I	1	24	7	34	18	49
Skeletal muscle, Calpain II	1	8	6	19	17	28

Calpain was purified from the indicated rat tissues as described in Methods. Calpain activity was assayed in the presence of 5 μM Ca^{2+} and, where indicated, of 50 μg/ml of membranes (17, 18).

DISCUSSION

The reported results, extending the previous observation on calpain from human neutrophils (12), show that IVC acts as a specific activator of some calpain forms present in various rat tissues. The activating action is absent on calpains I isolated from kidney, liver, heart and skeletal muscle (Fig. 1A), is maximal on calpains II from the same tissues (Fig. 1C) and is intermediate on calpain I from erythrocytes (Fig. 1B). Namely the sensitivity to Ca^{2+} of calpains II, the isoforms most susceptible to the activating action of IVC, is increased by approximately one order of magnitude. This class of proteinases is known to undergo autoproteolytic conversion to a low Ca^{2+} requiring form. Since autolysis requires high Ca^{2+} concentrations, an increase in Ca^{2+} sensitivity is considered to represent the initial signal for calpain activation. The mechanism by which IVC activates calpains II has been established to involve increased affinity for Ca^{2+} (Fig. 1) concurrent with an accelerated autolytic process (Fig. 3B). Furthermore IVC potentiates the catalytic activity of autolyzed calpain

II (Fig. 3B) possibly inducing favourable conformational changes of the enzyme. The lack of action of IVC on calpains I from different tissues, except erythrocytes, reflects the peculiar nature of this class of calpains which, unlike calpains II, are active at physiologically attainable Ca^{2+} concentrations independently on a previous autolysis. The definite but lower action of IVC on calpain I from rat erythrocytes (Fig. 1) and human neutrophils (12), the only isoform present in both these blood cells can be interpreted as due to an increased sensitivity to Ca^{2+} (Fig. 1B) resulting from a reversible conformational modification of the native enzyme.

The present results also indicate that IVC exhibits on both erythrocyte calpain I and tissutal calpains II an activating effect which is synergistic to that caused by phospholipid vesicles (Table I). The described action of IVC, an intermediate in leucine catabolism (19), is physiologically relevant for two reasons: its specificity and the relatively low concentrations at which it is active, conceivably approaching those attainable in cells under conditions wherein branched-chain amino acids are recruited as additional fuel (20).

It is tempting to speculate that activation of calpains by IVC may provide a mechanism for the initiation of controlled proteolysis in which also leucine catabolites are involved.

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