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The calmodulin-binding domain as an endogenous inhibitor of the *p*-nitrophenylphosphatase activity of the Ca^{2+} pump from human red cells

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Digestion of red cell membranes with chymotrypsin elicited *p*-nitrophenylphosphatase activity. During digestion, the *p*-nitrophenylphosphatase appeared in parallel with the activation of the Ca^{2+}-ATPase (in the absence of calmodulin). The chymotrypsin-activated *p*-nitrophenylphosphatase was inhibited by C20W, a 20 amino acid peptide modelled after the sequence of the calmodulin-binding site of the red cell Ca^{2+} pump (Vorherr et al. (1990) Biochemistry 29, 355–365). On the contrary, the ($ATP + Ca^{2+}$)-dependent *p*-nitrophenylphosphatase activity of intact red cell membranes was not affected by C20W. Ca^{2+} inhibited the chymotrypsin-induced *p*-nitrophenylphosphatase (K_i for $Ca^{2+} = 2$ μM). In the absence of ATP, C20W and Ca^{2+} did not interact in apparent affinity as inhibitors of this activity. On the other hand, in the presence of 2 mM ATP, Ca^{2+} antagonized the inhibition produced by C20W. The results are consistent with the idea that the calmodulin-binding site is an 'autoinhibitory domain' of the Ca^{2+} pump, and that removal of this domain by proteolysis, or its modification by calmodulin binding is the reason for the activation of both the ATPase and the *p*-nitrophenylphosphatase activity of the pump. The results presented in this paper give new information about the mechanism of the two kinds of *p*-nitrophenylphosphatase and about the nature of the apparent competition between C20W and Ca^{2+}.

Introduction

It is known that the *p*-nitrophenylphosphatase activity of the Ca^{2+} pump can be elicited by the Ca^{2+}-calmodulin complex [1] or by limited trypsinolysis of the enzyme [2]. Since limited proteolysis of the pump activates the ATPase activity of the pump in a calmodulin-like way, the stimulation of the *p*-nitrophenylphosphatase activity by proteolysis was attributed to a simulation of the effect of calmodulin [2].

Structural studies have revealed that proteolysis with either trypsin or chymotrypsin removes the calmodulin-binding domain of the pump. However, in addition to the C-terminal portion in which the calmodulin-binding domain is located, tryptic digestion removes a 30 kDa fragment from the N-terminal end of the molecule [3]. Chymotrypsin is more selective for the C-terminal end [4].

Recently it was demonstrated that synthetic peptides modelled after the sequence of the calmodulin-binding domain of the Ca^{2+} pump inhibited the pump after it had been treated with either trypsin or chymotrypsin, restoring to it the kinetic behavior of the intact system in the absence of calmodulin [5]. These results suggested that the calmodulin-binding domain is an 'autoinhibitory domain' and that either calmodulin binding or proteolytic attack modify this domain to suppress the inhibition. The activation of the *p*-nitrophenylphosphatase activity by limited proteolysis with trypsin [2] led us to the idea that the same concept could be applied to the *p*-nitrophenylphosphatase.

To test the above mentioned hypothesis: (1) we treated the enzyme with chymotrypsin instead of with trypsin. (2) we tested the effect of C20W, a synthetic peptide that follows the sequence of the calmodulin-binding domain of the Ca^{2+} pump [6,7]. The results

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are consistent with the idea that the calmodulin-binding domain is also autoinhibitory for the *p*-nitrophenylphosphatase activity of the pump.

Materials and Methods

Fresh blood from hematologically normal adults collected with acid citrate/dextrose solutions was always used. Red cell membranes were prepared following the procedure of Gietzen et al. [8], which yields membranes devoid of endogenous calmodulin. Calmodulin was purified from bovine brain as described by Kakiuchi et al. [9].

For treatment with chymotrypsin the membranes were washed and suspended in media containing: 120 mM KCl, 30 mM Tris-HCl (pH 7.4 at 37 °C) and 10 μg/ml of Na-*p*-tosyl-l-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin (60 units/mg). Unless otherwise indicated, the mixture was incubated at 4 °C and after 2 min chymotrypsin action was terminated by the addition of soybean trypsin inhibitor (final concentration: 200 μg/ml). Phosphatase activity was measured estimating the release of *p*-nitrophenol from *p*-nitrophenylphosphate [10]. All assays were carried out in polyethylene tubes. Except otherwise indicated in Results, the incubation media contained: 120 mM KCl, 6.25 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4 at 37 °C), 10 mM *p*-nitrophenylphosphate, 1.0 mM EGTA, 1 mM ouabain, 50–80 μg/ml membrane protein and the concentration of $CaCl_2$ necessary to give the free Ca^{2+} concentrations indicated in the legends of the figures. Ca^{2+}-ATPase activity was measured at 37°C in similar media to those used for the estimation of the phosphatase activity, but without the addition of *p*-nitrophenylphosphate and with a final concentration of 2 mM ATP. The concentration of $MgCl_2$ in such media was 3.75 mM. The release of inorganic phosphate from the nucleotide was estimated by a modification of the procedure of Fiske and SubbaRow [11]. To estimate the Ca^{2+}-dependent activities, the activities measured in similar media to those described above, but without the addition of $CaCl_2$ were subtracted. Free Ca^{2+} concentration in the incubation media was measured with an IS-561 Ca^{2+}-electrode [12]. Protein was estimated by the method of Lundahl [13].

Except where otherwise indicated, the experiments presented under Results were chosen as representative of two to four experiments. Each of the measurements was performed in triplicate and the individual values did not differ from the mean more than 10%. Equations were adjusted to the experimental results by least-squares non-linear regression, by using the algorithm of Gauss-Newton with optional damping [14]. The concentration variables were assumed to have negligible error and the variance of the velocity variable was assumed to be constant. Calculations were performed with a microcomputer Epson Equity III + . The program used allows the fitting of any function with up to two independent variables and up to 15 adjustable parameters and their standard deviation [15].

C20W was synthesized and purified as described [7]. ATP, TLCK-treated α-chymotrypsin and soybean trypsin inhibitor were obtained from Sigma (U.S.A.). Salts and reagents were of analytical reagent grade.

Results and Discussion

Effect of chymotryptic digestion on the Ca^{2+}-ATPase and the p-nitrophenylphosphatase activities of red cell membranes

Fig. 1 (A and B) shows the effect of chymotryptic digestion of red cell membranes on the ATPase and *p*-nitrophenylphosphatase activity of the Ca^{2+} pump. It can be seen (Fig. 1A) that 2 min of chymotryptic digestion suffices to activate maximally the Ca^{2+}-ATPase to a value which is 80% of that reached in the presence of 312 nM calmodulin. Further digestion produced a slight decrease in the activity. The Ca^{2+}-

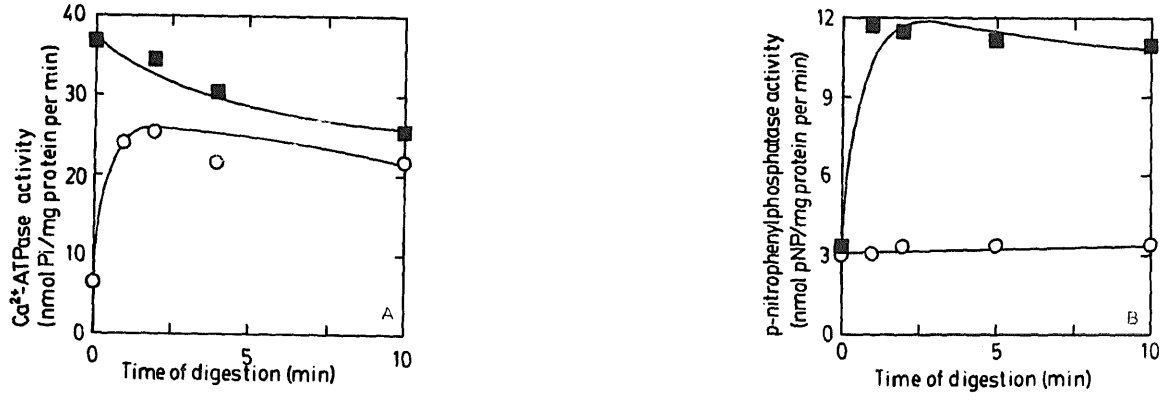

Fig. 1. (a) Ca^{2+}-ATPase activity as a function of time of digestion of the red cell membranes with chymotrypsin. Ca^{2+}-ATPase activity was measured in the absence (open circles) and in the presence (filled squares) of 312 nM calmodulin. (b) *p*-Nitrophenylphosphatase activity as a function of time of digestion of the red cell membranes with chymotrypsin. *p*-Nitrophenylphosphatase activity was measured in the presence (open circles) and in the absence (filled squares) of 50 μM Ca^{2+}.

ATPase activity in the presence of calmodulin slightly decreased during digestion to a value which, at 10 min of digestion, is about 70% of the activity of the undigested ATPase. Fig. 1B shows that 2 min of chymotryptic digestion suffice to activate the *p*-nitrophenylphosphatase activity assayed in the absence of Ca^{2+}, and that further digestion did not affect significantly this activity. This procedure did not modify the phosphatase activity of the membranes when it was measured in the presence of 50 μM Ca^{2+}. The results of Fig. 1 suggest that activation of the phosphatase in the absence of Ca^{2+} and stimulation of the calmodulin-independent Ca^{2+}-ATPase activity are simultaneous phenomena. The lack of effect of chymotryptic digestion on the *p*-nitrophenylphosphatase activity assayed in the presence of 50 μM Ca^{2+} suggests that, as was stated before [2], Ca^{2+} inhibits completely the *p*-nitrophenylphosphatase activity elicited by proteolysis.

Effect of C20W on the p-nitrophenylphosphatase activity of intact and chymotrypsin-treated membranes

The fact that activation of the *p*-nitrophenylphosphatase in the absence of Ca^{2+} and stimulation of the Ca^{2+}-ATPase activity in the absence of calmodulin are simultaneous phenomena suggests that both are consequences of the same event. Since chymotrypsin cleaves the C-terminal part of the Ca^{2+} pump molecule, where the calmodulin-binding domain is located [6], it is tempting to suggest that the deletion of the calmodulin-binding domain is the reason for the observed results. To test this hypothesis, we used a synthetic peptide, named C20W, which was synthesized following the amino acid sequence of the calmodulin-binding domain of the Ca^{2+} pump [7]. The *p*-nitrophenylphosphatase activity of chymotrypsin-treated membranes was then measured as a function of C20W concentration. Activity in the presence of 50 μM Ca^{2+} was not affected by the peptide and was subtracted from the total activity. Fig. 2 shows that C20W completely inhibited the *p*-nitrophenylphosphatase along a hyperbolic curve. The apparent K_i value was 13.1 ± 3.5 μM. This value is similar to that reported for C20W as inhibitor of active Ca^{2+} transport in chymotrypsin-treated IOVs [5], suggesting that both *p*-nitrophenylphosphatase activity and active Ca^{2+} transport are inhibited by binding of C20W to the same site in the pump. In the same figure it is shown that (Ca^{2+} + ATP)-dependent *p*-nitrophenylphosphatase activity of intact membranes was not affected at all by C20W, indicating that inhibition is evident for the *p*-nitrophenylphosphatase activity of digested membranes only. As the Ca^{2+} pump from intact membranes already possesses the calmodulin-binding domain in its structure, the lack of effect of C20W on the (Ca^{2+} + ATP)-dependent *p*-nitrophenylphosphatase activity of intact membranes was the expected result. On the other hand, the sensitivity

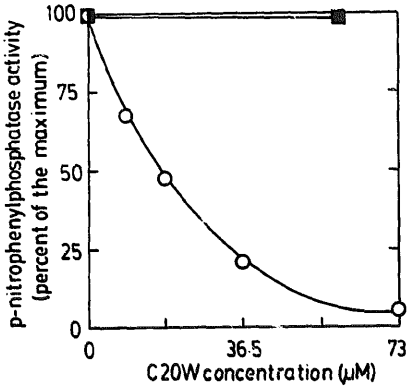

Fig. 2. *p*-Nitrophenylphosphatase activity of chymotrypsin-treated membranes (open circles) and (Ca^{2+} + ATP)-dependent *p*-nitrophenylphosphatase activity of intact membranes (filled squares) as a function of C20W concentration. In the case of *p*-nitrophenylphosphatase activity of chymotrypsin-treated membranes the activity in the presence of 50 μM Ca^{2+} was subtracted. In the case of (Ca^{2+} + ATP)-dependent *p*-nitrophenylphosphatase activity of intact membranes, ATP was 2 mM, free Ca^{2+} was 50 μM and the activity in the absence of Ca^{2+} was subtracted. 100% was 5.3 ± 0.4 nmol/mg of protein per min for the activity of chymotrypsin-treated membranes and 5.8 ± 0.4 nmol/mg of protein per min for (Ca^{2+} + ATP)-dependent activity of intact membranes.

of the *p*-nitrophenylphosphatase activity elicited by chymotryptic digestion to C20W confirms that this activity belongs to the Ca^{2+} pump and supports the idea that the calmodulin-binding domain is an endogenous inhibitor of both the ATPase activity in the absence of calmodulin [5] and the *p*-nitrophenylphosphatase activity of the Ca^{2+} pump in the absence of Ca^{2+}.

The calmodulin-binding domain was identified as an endogenous inhibitor of other enzymes regulated by calmodulin, like the myosin light chain kinase [17] and the calmodulin kinase II [18]. However, in the latter case the calmodulin-binding domain and the autoinhibitory domain do not overlap completely [19].

Effect of Ca^{2+} on the inhibition by C20W

The phosphatase activity induced by proteolysis was previously shown to be inhibited by Ca^{2+} [2]. As C20W inhibition of the proteolysed Ca^{2+} pump is competitive with Ca^{2+}, it was interesting to know whether C20W (the calmodulin-binding site) had any effect on the apparent affinity for Ca^{2+} as inhibitor of the *p*-nitrophenylphosphatase. This was tested by measuring the *p*-nitrophenylphosphatase activity induced by chymotrypsin as a function of Ca^{2+} concentration in the absence and in the presence of 10 μM C20W (Fig. 3). It can be seen that both in the absence and in the presence of the peptide, Ca^{2+} abolished the phosphatase activity elicited by the chymotrypsin treatment. It can also be seen that 10 μM C20W inhibited this activity by about 30% at all the Ca^{2+} concentrations tested but 10 μM Ca^{2+}. It should be pointed out that

at 10 μM Ca^{2+} the activity is very low, so the relative error of the experimental values are greatly increased. K_i values for Ca^{2+} were 2.1 ± 0.3 μM in the absence and 2.2 ± 0.3 μM in the presence of 10 μM C20W, whereas V_i (the activity inhibitable by Ca^{2+}) dropped from 5.6 ± 0.3 nmol/mg of protein per min in the absence to 4.1 ± 0.2 nmol/mg of protein per min in the presence of 10 μM C20W. In a separate set of experiments (not shown) we estimated the apparent affinity of C20W as inhibitor of the chymotrypsin-activated *p*-nitrophenylphosphatase at different Ca^{2+} concentrations from 0 to 3.84 μM. The apparent value of K_i for C20W did not change significantly with Ca^{2+} concentration ($K_i = 11.0 \pm 0.7$ μM). On the other hand, the activity in the absence of peptide fell from 6.3 ± 0.1 nmol/mg of protein per min to 2.6 ± 0.1 nmol/mg of protein per min at 3.84 μM Ca^{2+}. From these results and from those of the experiment of Fig. 3 it can be concluded that, under the conditions described, C20W and Ca^{2+} do not interact in affinity as inhibitors of the chymotrypsin-induced *p*-nitrophenylphosphatase. Therefore, the effect of C20W on the Ca^{2+} dependence of the *p*-nitrophenylphosphatase activity was checked in ATP containing media. In Fig. 4 is shown the result of an experiment in which *p*-nitrophenylphosphatase activity of chymotrypsin-treated membranes was measured as a function of Ca^{2+} in media containing 2 mM ATP, in the absence and in the presence of 10 μM C20W. At variance with what is observed in the absence of ATP, in the presence of the nucleotide Ca^{2+} did not produce inhibition, but a small activation of the *p*-nitrophenylphosphatase. This is consistent with what was previously observed for the *p*-nitrophenylphosphatase activity of trypsin-treated membranes [2]. At 0 μM Ca^{2+}, 10 μM C20W inhibited the *p*-nitrophenylphosphatase by 25%. As Ca^{2+} concentration is increased, inhibition of the *p*-nitrophenylphosphatase activity is progressively overcome, and at 8 μM Ca^{2+} the effect of C20W is no longer detectable.

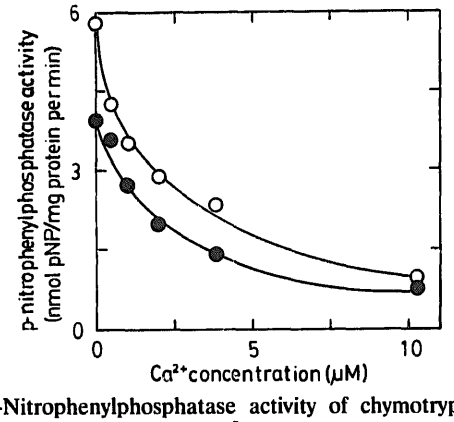

Fig. 3. *p*-Nitrophenylphosphatase activity of chymotrypsin-treated membranes as a function of Ca^{2+} concentration in the absence (open circles) and in the presence (filled circles) of 1.0 μM C20W. The activity in the presence of 50 μM Ca^{2+} was subtracted.

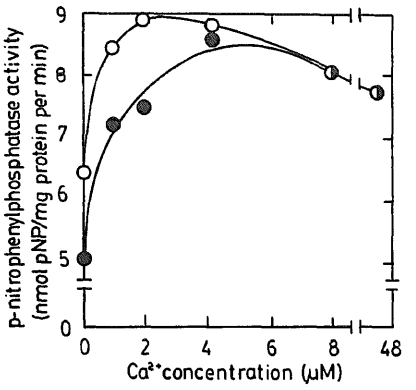

Fig. 4. *p*-Nitrophenylphosphatase activity of chymotrypsin-treated membranes as a function of Ca^{2+} concentration in the absence (open circles) and in the presence (filled circles) of 10 μM C20W. All media contained 2 mM ATP. Total *p*-nitrophenylphosphatase activity was represented.

Thus, unlike in its absence, in the presence of 2 mM ATP Ca^{2+} antagonized the effect of C20W on the *p*-nitrophenylphosphatase activity. For the interpretation of these results it is important to consider that in the presence of Ca^{2+} and ATP, the pump is making the complete Ca^{2+} transport cycle, while in the absence of these substrates this cycle cannot occur. The *p*-nitrophenylphosphatase measured in the presence of Ca^{2+} and ATP appears to be a result of the complete cycle. In the absence of ATP the unphosphorylated conformers of the enzyme are distributed in equilibrium. In such conditions, a *p*-nitrophenylphosphatase that requires removing or modification of the calmodulin-binding domain is elicited. The data reported here confirm that this *p*-nitrophenylphosphatase occurs by a different mechanism.

The fact that, in the absence of ATP, C20W and Ca^{2+} did not interact in affinity during the inhibition of the *p*-nitrophenylphosphatase suggests that a ternary complex enzyme-C20W-Ca^{2+} is formed. When the pump is making the complete Ca^{2+} transport cycle (in the presence of ATP) since Ca^{2+} overcame the inhibition by C20W, such a complex would not be formed. Probably the reason for this is that binding of Ca^{2+} is slowed by C20W and conversely, binding of C20W is slowed by Ca^{2+}.

An alternative explanation for these results is that, for inhibition of the *p*-nitrophenylphosphatase, Ca^{2+} binds to a different site than the Ca^{2+} transport site. However, since the apparent affinity for Ca^{2+} during both phenomena is similar and it changes in similar way in response to acidic phospholipids [22] this interpretation seems rather unlikely.

In summary, the lack of competition between Ca^{2+} and C20W observed in the absence of ATP indicates that the proteolytically activated *p*-nitrophenylphosphatase proceeds by a different mechanism in the absence of ATP than in its presence, and suggests that

the apparent competitive effects between Ca^{2+} and C20W observed in Ca^{2+} uptake and in *p*-nitrophenylphosphatase measured in the presence of ATP are derived from kinetic interactions rather than from physical competition for a binding site in the pump.

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