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Dipeptidyl peptidase IV of human lymphocytes

Evidence for specific hydrolysis of glycylproline *p*-nitroanilide in T-lymphocytes

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Glycylproline *p*-nitroanilide is hydrolysed in lymphocytes from human blood exclusively by dipeptidyl peptidase IV. This was demonstrated by specific inhibition with *N*-alanylprolyl-*O*-(4-nitrobenzoyl)hydroxylamine and di-isopropyl phosphorofluoridate and by studying the membrane localization of dipeptidyl peptidase IV and determining specific dipeptidyl peptidase II activity. Additional evidence that dipeptidyl peptidase IV is a marker for T-lymphocytes, obtained from determinations of biochemical activity on intact lymphocyte preparations and correlation studies with other T-cell markers, is also presented.

Dipeptidyl peptidase IV (DP IV, EC 3.4.14.–) is known to be an enzyme located in the plasma membrane of a variety of mammalian cells (for a review see Walter *et al.*, 1980). With the use of Gly-Pro-methoxy-β-naphthylamide as substrate for histochemical demonstration, this enzyme was also detected in lymphatic tissue and in smears of isolated lymphocytes (Lojda, 1977; Gossrau, 1981; Feller, 1982; Chilosi *et al.*, 1982). Recently, evidence for the T-cell origin of DP IV in separated MNC was provided by Feller *et al.* (1982). However, the possibility that Gly-Pro-Xaa hydrolysis demonstrated in lymphocytes might be due to DP II or other peptidases was not excluded.

The present paper gives evidence that DP IV is the only enzyme responsible for Gly-Pro-pNA hydrolysis in human lymphocytes. This conclusion is drawn from (1) determination of pH optimum, (2) comparison of hydrolysis of substrates for DP II and DP IV, (3) confirmation of the plasma-membrane localization of the enzyme, and (4) specific inhibition of enzyme activity.

Additional evidence in favour of the supposed T-cell origin of DP IV (Feller, 1982) is provided by determinations of biochemical activity on non-lysed intact T-cells and malignant chronic-lymphocytic-leukaemia (B-cell type) lymphocytes and by correlating DP IV activity with known T-cell markers.

Abbreviations used: DP II and DP IV, dipeptidyl peptidases II and IV; E-rosettes, rosettes formed between lymphocytes and sheep erythrocytes; MNC, mononuclear leucocytes from venous blood; -pNA, *p*-nitroanilide.

Materials and methods

The MNC were prepared from peripheral venous blood by using the procedure described by Bøyum (1968) with minor modifications. For routine determinations MNC were adjusted to 3×10^6 cells/ml in 0.15M-potassium phosphate buffer, pH 7.6 (DP IV reaction buffer). T-lymphocytes were enriched by rosetting of T-lymphocytes with *S*-2-aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocytes as described by Madsen *et al.* (1980). T- and non-T-cells were washed thrice and resuspended in potassium phosphate buffer for DP IV reaction. To determine the T-cell content of MNC preparations, the E-rosette test was modified to a micro scale (1.5×10^5 MNC, 7.5×10^4 *S*-2-aminoethylisothiouronium bromide hydrobromide-treated sheep erythrocytes and 1.5% human serum albumin per tube) and the rosettes were counted after overnight incubation at 4°C.

As an alternative method for determination of the T-lymphocyte content, smears of MNC preparations were stained for unspecific acid α-naphthyl acetate esterase. For this purpose the method described by Horwitz *et al.* (1977) was slightly modified. Counterstaining was performed with Methyl Green.

Monocytes were enriched by adherence (2h, 37°C) on glass or polystyrene Petri dishes. MNC were also prepared from peripheral blood of patients with chronic lymphocytic leukaemia of B-cell origin (as established by cell marker and clinical diagnosis).

Enzyme activity of DP IV was determined by incubating 3×10^5 MNC in 100 μl of DP IV reaction buffer with 100 μl of 0.6 mM-Gly-Pro-pNA or -Ala-Pro-pNA for 120 min at 37°C. The reaction was stopped by adding 400 μl of 1 M-sodium acetate buffer, pH 4.5, to 100 μl of incubation mixture. The absorbance of the 6000*g*-min supernatant was measured at 390 nm. Protein determinations were carried out by using a micro modification (Langner *et al.*, 1971) of the method inaugurated by Lowry *et al.* (1951); human serum albumin was used as standard.

Results and discussion

Xaa-Pro derivatives can be hydrolysed by two different dipeptidyl peptidases, namely DP IV and DP II (Walter *et al.*, 1980; McDonald & Schwabe, 1977, 1980). To decide which of them is responsible for degradation of Gly-Pro-pNA in human lymphocytes, the following experiments were carried out.

Firstly, studying the pH-dependence of $k_{cat.}/K_m$, we found the optimum of enzymic Gly-Pro-pNA hydrolysis in lymphocytes to be in the pH range 7.4–7.6 (results not shown). These values correspond to those obtained for DP IV from other organs under comparable conditions (Walter *et al.*, 1980; Küllertz *et al.*, 1981; Schön, 1983). DP II, however, shows an optimum in the pH range 5–6 with the same substrate, as well as with the DP II-specific substrate Lys-Ala-Xaa, under conditions of $[S] \gg K_m$ (McDonald & Schwabe, 1980; Fukasawa *et al.*, 1983; Sannes, 1983).

Secondly, the hydrolysis rates of Lys-Ala-pNA, the substrate preferred by DP II (McDonald & Schwabe, 1977, 1980), were less than 3% at pH 7.6 and less than 4% at pH 5.5 compared with the hydrolysis of Gly-Pro-pNA. In the light of the substrate-specificity of DP II and DP IV (Lys-Ala-Xaa is hydrolysed by DP II 20-fold faster than Gly-Pro-Xaa; McDonald & Schwabe, 1977), human lymphocytes possess only a very low DP II activity, which does not appear to contribute to the hydrolysis of Gly-Pro-pNA. The K_m value for Gly-Pro-pNA hydrolysis in a Triton extract of human lymphocytes at pH 7.4 was determined as 0.16 ± 0.03 mM, which is comparable with values reported for DP IV from rat liver (Hopsu-Havu & Sarimo, 1967) and pig kidney (Macnair & Kenny, 1979). Fukasawa *et al.* (1983) reported a K_m value for DP II with the same substrate of 0.1 mM.

Thirdly, experimental evidence on the supposed plasma-membrane localization of Gly-Pro- peptidase was obtained. In three separate experiments no increase in enzyme activity was observed after lysing freshly prepared (more than 95% viable) lymphocytes with 0.2% Triton X-100 or freezing and thawing them five times. So it is concluded that intracellular enzymes do not contribute to Gly-Pro-pNA hydrolysis in lymphocytes. As in other mammalian cells (Kenny *et al.*, 1976; Elovson, 1980; Gossrau, 1981; Schön, 1983), DP IV appears to be localized in the plasma membrane of human lymphocytes as an ectoenzyme. In contrast with DP IV, DP II is localized in the lysosome (McDonald & Schwabe, 1980; Sannes, 1983), although Feller *et al.* (1982) suggested a lysosomal origin of Gly-Pro-Xaa-degrading activity in lymphocytes. However, it is probable that the lysosomal fractions used by them contained substantial amounts of plasma-membrane vesicles, which are very similar in size and density to lysosomes.

From these data it is suggested that DP IV, but not DP II, is responsible for the degradation of Gly-Pro-pNA in human lymphocytes.

Fig. 1 demonstrates that enzyme activity was completely inhibited by the alkylating reagent di-isopropyl phosphorofluoridate at a molar ratio $[I]/[E_{app.}] = 1.2 \times 10^6$ as well as by an enzyme-activated suicide inhibitor that is effective against DP II and DP IV (Fischer *et al.*, 1983), *N*-Ala-Pro-*O*-(4-nitrobenzoyl)hydroxylamine, at a ratio of $[I]/[E_{app.}] = 5 \times 10^6$ {where [I] represents the concentration of inhibitor and $[E_{app.}]$ represents the apparent concentration of active centres of DP IV assuming the $k_{cat.}$ value of $110\,s^{-1}$ as determined for the pig kidney enzyme (G. Fischer, unpublished work) because no data exist about the purified enzyme from human lymphocytes}. From these inhibitor data the possibility is excluded that Gly-Pro-pNA is hydrolysed in human lymphocytes by other peptidases than Xaa-Pro-specific dipeptidyl peptidases with a serine residue in the active centre. In contrast with our results, Feller *et al.* (1982) did not find inhibition of cytochemically reactive DP IV by di-isopropyl phosphorofluoridate, which was misinterpreted as an argument against its identity as DP II. However, DP II is known to be a serine enzyme, too (McDonald & Schwabe, 1980).

Feller *et al.* (1982) used a Triton X-100 extract of a pellet containing the majority of the lysosomes for determination of DP IV activity in different lymphocyte populations by a fluorimetric assay. The DP IV extraction procedure, however, can be accompanied by losses or inactivation of enzyme. Therefore we used a direct DP IV assay by incubating intact, viable, MNC in suspension with

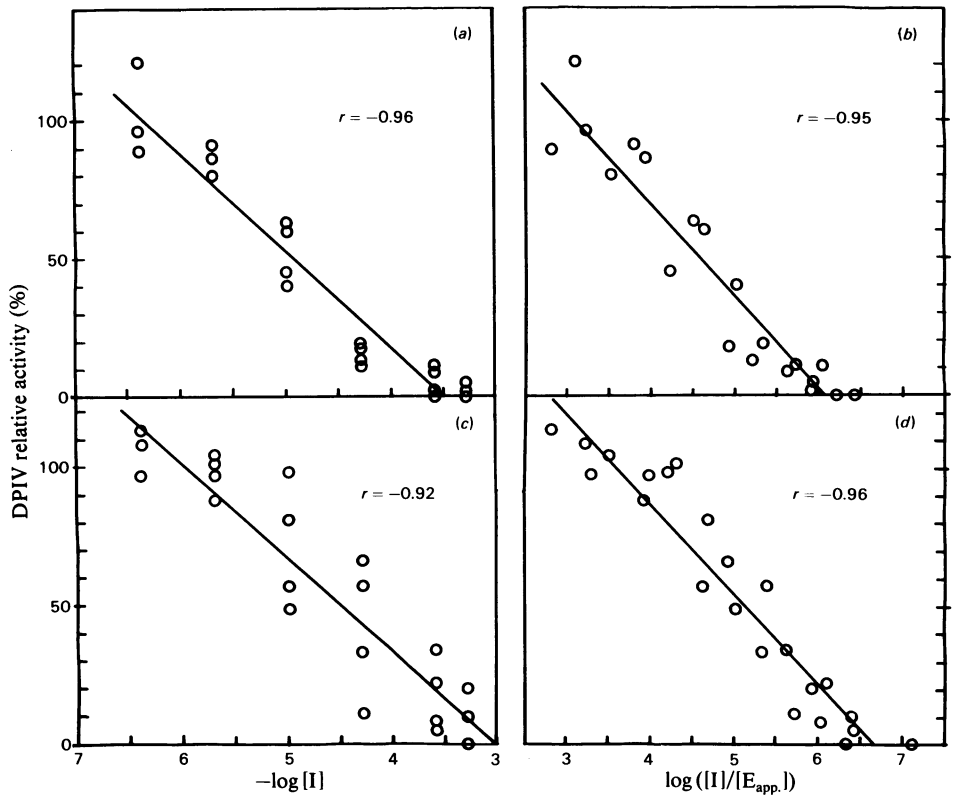

Fig. 1. *Inhibition of Gly-Pro-pNA hydrolysis in human lymphocytes by di-isopropyl phosphorofluoridate (a and b) and N-Ala-Pro-O-(4-nitrobenzoyl)hydroxylamine (c and d)*

Enzyme activity was plotted against the inhibitor concentration during preincubation (*a* and *c*) and against the concentration ratio inhibitor/active centres in the control (*b* and *d*). Data were plotted by using a program for optimization of linear regression. Each point is the result of triplicate determinations. Lymphocytes were preincubated with inhibitor for 2h at 37°C; the final concentration of Gly-Pro-pNA was 4mM. [I], inhibitor concentration (preincubation); $[E_{app.}]$, concentration of active centres assuming a $k_{cat.}$ value of $110\,s^{-1}$ as determined for the pig kidney enzyme; *r*, slope of the regression line.

Gly-Pro-pNA. As shown in Table 1, the specific activity of DP IV, related either to cell number or to protein, was significantly higher in T-lymphocyte preparations and lower in adherent cells, which consisted mainly of monocytes and B-lymphocytes, or in MNC depleted of T-lymphocytes. Furthermore, MNC obtained from patients with chronic lymphocytic leukaemia of B-cell type exhibited very low DP IV activities, corresponding to a low T-cell content (compare the values for E-rosettes in Table 1). Similar observations were reported by Chilosi *et al.* (1982) and Feller *et al.* (1982), with the use of other methods.

A direct correlation between DP IV activity and percentage of T-lymphocytes in MNC from peripheral blood had not previously been established. Therefore, we compared the enzyme activity with the T-cell content in different MNC preparations from healthy volunteers (including T-cell and monocyte preparations) and found good correlation between Gly-Pro-pNA hydrolysis on the one hand and E-rosette percentage ($r_s = 0.6$, $P < 0.001$, $n = 61$) or percentage of (unspecific acid α-naphthyl acetate esterase)-positive (focal and granular reaction) T-cells ($r_s = 0.4$, $P < 0.027$, $n = 34$) on the other hand. This direct correlation of independent markers provides an additional proof for the specificity of DP IV as a T-cell marker.

It may be noted that Feller *et al.* (1982) demonstrated that DP IV is localized mainly in T-cells with receptors for the Fc part of macroglobulin (T_M-lymphocytes). Therefore it would be of particular interest whether this enzyme plays a role in

Table 1. *Activity of DP IV in human MNC populations*

MNC prepared from venous blood were further fractionated by using rosetting with sheep erythrocytes into T-cell-enriched (II) and T-cell-depleted (III) cell preparations or using adherence to glass or plastic surfaces into adherent (V) and non-adherent (IV) MNC; MNC from peripheral blood of patients with chronic lymphocytic leukaemia of B-cell origin (B-CLL) were also examined. Values are given as means ± S.E.M. (*n* experiments). Statistical differences between different groups were calculated by using the *H*-test procedure of Kruskal & Wallis. The significance level in all cases was at least 1%. Index[a] refers to the first column, and index[b] refers to the second column of enzyme activity.

Cell population	T-lymphocyte content (E-rosettes, %)	DP IV activity (pkat/10^6 MNC)	DP IV activity (pkat/ng of protein)	Statistics: difference against population
I MNC	79±5 (62)	3.4±0.1 (62)	65±3 (50)	
II T-cell-enriched	90±2 (15)	4.5±0.4 (15)	135±12 (12)	I^b, $III^{a,b}$
III T-cell-depleted	33±5 (15)	1.7±0.2 (15)	41±5 (15)	$I^{a,b}$, $II^{a,b}$
IV Non-adherent cells	61±6 (6)	3.6±0.4 (6)	149±21 (6)	I^b, V^a
V Adherent cells	14±6 (6)	1.2±0.2 (6)	28±9 (3)	I^a, IV^a
VI MNC from B-CLL	15±2 (16)	1.1±0.1 (16)	36±7 (11)	I^a

the expression of regulatory functions in different T-lymphocyte subsets.

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