Activation of glucose-6-phosphatase in intact hepatic microsomes

There have been three recent reports from independent laboratories that calcium activates glucose-6-phosphatase activity in rat intact liver microsomes (Yamaguchi *et al.*, 1989; Waddell *et al.*, 1990; Mithieux *et al.*, 1990). Glucose-6-phosphatase activity in intact rat liver microsomes is a measure of the combined rates of the glucose-6-phosphatase enzyme and its three transport proteins T_1, T_2 and T_3, which respectively transport glucose-6-phosphate, phosphate and pyrophosphate, and glucose across the endoplasmic reticulum membrane (see Fig. 1). In addition, a Ca^{2+}-binding regulatory protein is also essential for normal glucose-6-phosphatase activity (see Fig. 1). Ca^{2+} could therefore affect any one of the five proteins. Recently Waddell *et al.* (1990) demonstrated, using the alternative substrate pyrophosphate, that the transport protein T_2 was the component of glucose-6-phosphatase upon which Ca^{2+} has the greatest effect in intact microsomes.

In complete contrast, van de Werve (1989) concluded that Ca^{2+} inhibits glucose-6-phosphatase activity in intact rat liver microsomes. In this case the glucose-6-phosphatase activity in the presence of 25 mM-EGTA was only compared to the activity of glucose-6-phosphatase in the presence of 25 mM-EGTA plus various amounts of Ca^{2+}. Essential control data on the activity of glucose-6-phosphatase without any additions, in the presence of Ca^{2+} alone or in the presence of other similar metal chelators like EDTA were not given.

In our recent paper (Waddell *et al.*, 1990) we studied the effects of Ca^{2+} on T_2 in the absence of EGTA because we found that high EGTA concentrations had large effects on glucose-6-phosphatase activity in both intact and disrupted microsomes, making interpretation of data very complex. As early as 1983, EGTA was reported to have inhibitory effects on glucose-6-phosphatase activity (Yamaguchi & Momose, 1983) although how this effect was manifested was unclear. Recently however, Mithieux *et al.* (1990) carried out a series of very elegant studies of the effects of Ca^{2+}, EGTA and Ca^{2+}–EGTA complexes on glucose-6-phosphatase activity. They not only confirmed that the activity of the glucose-6-phosphatase in intact liver microsomes can be activated by Ca^{2+}, they also showed that glucose-6-phosphatase activity can be decreased in the presence of Ca–EGTA complex, but that free Ca^{2+} plays no role in the inhibition which is caused by the presence of Ca–EGTA complex.

It is therefore most surprising that, in the preceding letter, van de Werve & Vidal (1990) still conclude "...it is clear that in our experimental conditions, EGTA can indeed be used to assess the effects of Ca^{2+} on glucose-6-phosphatase...". We cannot agree, as the careful reappraisal of the effects of Ca^{2+} and EGTA by Mithieux *et al.* (1990) clearly demonstrate that the effects of Ca^{2+} alone and Ca^{2+}–EGTA complex are quite different.

In the preceding letter (van de Werve & Vidal, 1990), a number of other more methodological points are also raised. It is suggested that differences in buffers or pH of the assay might account for the differences in the results reported by Waddell *et al.* (1990) and by van de Werve (1989). This is unlikely as Mithieux *et al.* (1990) tested both assay conditions and obtained the same effects of Ca^{2+} and EGTA. It is also stated (van de Werve & Vidal, 1990) that "...the duration of the incubation might be critical in the assay of the enzyme in the presence of EGTA in disrupted microsomes", suggesting that during our 10 min assays glucose-6-phosphatase activity is not linear with time. This is not correct. Not only are our assays linear with time at 30 °C for at least 1 h when EGTA is present (as long as sufficient substrate is present), but 10 years ago (Table 1 in Burchell & Burchell, 1980) we clearly demonstrated that EGTA does not alter the stability of glucose-6-phosphatase in detergent-disrupted microsomes.

In the preceding letter, it is stated that the kinetic parameters (K_m and $V_{max.}$) of glucose-6-phosphatase measured in the presence of 25 mM-EGTA published in van de Werve (1989) were in the normal range reported by other workers in the field. This statement is somewhat misleading. It is true that some of the values are in the normal range but others are not. For example, the K_m values of the glucose-6-phosphatase enzyme in disrupted microsomes of 2.6 and 4.3 mM in the presence of 25 mM-EGTA and Ca^{2+} plus 25 mM-EGTA respectively are not normal. The normal K_m value is less than 1 mM (e.g. see Arion *et al.*, 1980; Waddell & Burchell, 1988). The most logical reason for the abnormally high K_m values reported by van de Werve is the fact that microsomes were disrupted using 0.4% deoxycholate, a detergent well known to inhibit glucose-6-phosphatase at such high concentrations (e.g. see Collilla *et al.*, 1974; Lange *et al.*, 1986). In contrast, we did not use an inhibitory negatively charged detergent to disrupt microsomes because of the many problems associated with studying the effects of Ca^{2+} in its presence. We used lower non-inhibitory concentrations of the non-ionic detergent Lubrol 12A-9.

There are similar problems with the measurements of intactness in van de Werve (1989). In the preceding letter it is stated that the latency of mannose-6-phosphatase activity in van de Werve (1989) in the presence of 25 mM-EGTA was 90%. Unfortunately, the way the mannose-6-phosphatase data is presented in Fig. 3 of that paper is a little misleading. The glucose-6-phosphatase activity value given in disrupted microsomes is 137 using 0.5 mM substrate and the mannose-6-phosphatase value using 10 times higher substrate (5 mM) is given as 153. Using the K_m value of either 4.3 or 2.6 mM, with a value of 137 at 0.5 mM, the activity

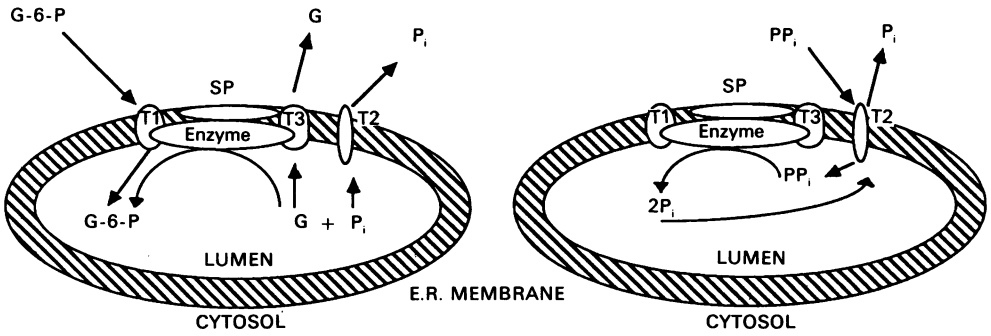

Fig. 1. The hepatic microsomal system

Key: G, glucose; G-6-P, glucose-6-phosphate; SP, stabilizing protein; E.R., endoplasmic reticulum.

at 5 mM should be several times higher than 153. The mannose-6-phosphatase activity reported is extremely low and abnormal, therefore it is not possible to accurately calculate intactness from the data given in Fig. 3 of van de Werve (1989). In the preceding letter it is stated that Mithieux *et al.* (1990) confirmed the measurements of intactness of van de Werve (1989) that were made in the presence of EGTA. This is not correct. Mithieux *et al.* (1990) demonstrated that their microsomal preparations were at least 97% intact. However they gave no values anywhere in their paper of mannose-6-phosphatase activity in the presence of 25 mM-EGTA.

In conclusion, the preceding letter does not add any new evidence to support the suggestion either that physiological Ca^{2+} inhibits glucose-6-phosphatase or that measuring glucose-6-phosphatase in the presence of 25 mM-EGTA is a valid method to study the effects of free Ca^{2+} on any of the proteins that comprise glucose-6-phosphatase..

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Angiotensin converting enzyme: how reliable is the fluorimetric assay with benzoyl-Gly-His-Leu as substrate?

For 20 years benozyl-Gly-His-Leu (BzGly-His-Leu) has been used as model substrate for the assay of the cell-surface peptidase angiotensin converting enzyme (ACE; EC 3.4.15.1) (Cushman & Cheung, 1971). The removal of the dipeptide His-Leu from the *C*-terminus of this tripeptide [Fig. 1, reaction (*a*)] is analogous to the action of ACE on its physiological substrate angiotensin I. Fluorescent based assays were designed to alleviate the need to extract the released BzGly in organic solvents and to increase the sensitivity by reacting the released His-Leu with either *o*-phthaldialdehyde (Yang & Neff, 1972) or fluorescamine (Conroy & Lai, 1978). Over the last two decades these fluorescent based assays have been widely used to measure ACE activity in a diverse range of samples, including serum, tissue extracts and cultured cells (see for example Friedland & Silverstein, 1976; Arregui & Iversen, 1978; Ehlers *et al.*, 1986; Dasarathy & Fanburg, 1989). However, with impure enzyme samples these fluorimetric assay methods based on the detection of the released His-Leu can give rise to erroneously low (and misleading) results (as discussed, for example, in Bourne *et al.*, 1989).

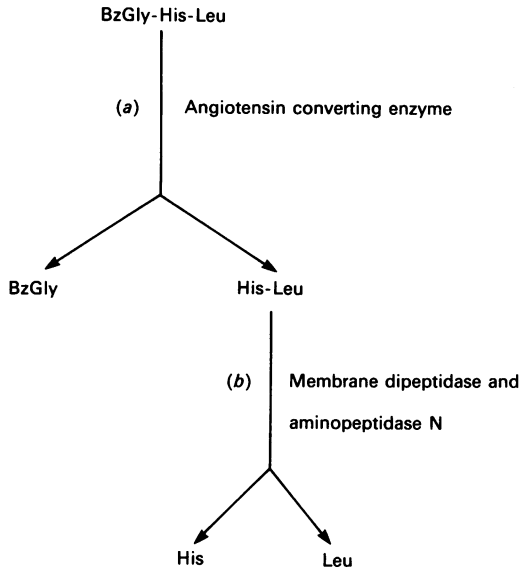

Fig. 1. Schematic representation of the complete metabolism of BzGly-His-Leu by (*a*) angiotensin converting enzyme and (*b*) other peptidases

This is because the dipeptide His-Leu is itself a substrate for other cell-surface peptidases [Fig. 1, reaction (*b*)] and during the course of the assay for ACE will be hydrolysed to its constituent amino acids which do not react significantly with *o*-phthaldialdehyde (Friedland & Silverstein, 1976) or fluorescamine (Udenfriend *et al.*, 1972). For example, His-Leu is rapidly hydrolysed by membrane dipeptidase (renal dipeptidase; dehydropeptidase-I; EC 3.4.13.11) at a rate of 54.5 μmol of His-Leu degraded/min per mg of purified pig kidney enzyme. This is compared with the specific activity of ACE towards BzGly-His-Leu of 9.41 μmol of BzGly produced/min per mg of purified pig kidney enzyme. It was actually by its ability to cleave the His-Leu released from BzGly-His-Leu by ACE that membrane dipeptidase was first identified as a glycosylphosphatidylinositol-anchored protein (Turner & Hooper, 1990). Another cell-surface peptidase, aminopeptidase N (EC 3.4.11.2), is also capable of cleaving His-Leu, at a rate of 0.62 μmol of His-Leu degraded/min per mg of purified pig kidney enzyme.

The inclusion of the specific inhibitors of membrane dipeptidase and aminopeptidase N, cilastatin (10 μM) and amastatin (10 μM) respectively, when assaying for ACE in pig kidney homogenates, leads to a 70% increase in fluorescence readings with *o*-phthaldialdehyde. These inhibitors do not interfere with the fluorimetric detection of His-Leu and have negligible effect on the activity of ACE towards BzGly-His-Leu. Membrane dipeptidase and aminopeptidase N are widely distributed in mammalian tissues, being particularly abundant in kidney and lung, tissues also rich in ACE (McDonald & Barrett, 1986; Turner & Hooper, 1989).

As early as 1971 whilst studying ACE in pig and guinea pig tissues it was observed that lung contains a considerable amount of a His-Leu hydrolysing activity (Lee *et al.*, 1971) which from the properties examined (molecular mass, lack of requirement for chloride ions and inhibition by metal chelators), was almost certainly membrane dipeptidase. It has also been shown that human serum also contains a dipeptide hydrolysing activity which could lead to an underestimation of ACE activity when using the fluorimetric assay (Friedland & Silverstein, 1976). More recently it has been shown that homogenates of bovine tissues contain enzymes that hydrolyse His-Leu, and, in this case, the inclusion of phenylmethanesulphonyl fluoride (1 mM) and *N*-