THE EFFECT OF VINBLASTINE ON THE GLUCAGON, BASAL AND GTP-STIMULATED STATES OF THE ADENYLATE CYCLASE FROM RAT LIVER PLASMA MEMBRANES

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1. Introduction

The *Vinca* alkaloid, vinblastine is often used as a tool to demonstrate the importance of microtubules in cellular events because it binds with high affinity to specific sites on tubulin causing the blockage of polymerisation [1–4]. Clearly for the action of such a tool to be interpreted correctly it must exhibit a high specificity.

Some of the events in which microtubules are purported to play a role are susceptible to changes in cyclic AMP levels [1,2,5,6]. Indeed, even the assembly of microtubules themselves is sensitive to cyclic nucleotides [1,2,4,7]. It is important then to appreciate the effects of vinblastine on the enzymes of cyclic AMP metabolism. This study examines the effect of vinblastine on the well-defined glucagon-stimulated adenylate cyclase from rat liver plasma membranes [8,9]. It dissects out its action on the basal and hormone-stimulated activities both in the presence and absence of the modulator GTP [8,9] which also has a central involvement in the polymerisation of microtubules [1–4].

2. Materials and methods

Plasma membranes were prepared from male Sprague-Dawley rats (200–300 g) and adenylate cyclase was assayed as detailed in [10]. Assays were performed at a final pH 7.4 with vinblastine present and at a plasma membrane concentration of 0.15 mg protein/ml. Vinblastine did not affect our method of assay of the cyclic AMP produced. ^{125}I-Specific glucagon binding to receptors was performed as in [11]. Protein estimation was by a modified microbiuret method [12].

Plasma membranes (3.25 mg/ml) were incubated with a range of rabbit antitubulin antibody (5–50 μg/ml) for 30 min at 4°C before aliquots were taken for assay.

Glucagon was a kind gift from Dr W. W. Bromer of Eli Lilly Co., Indiana. Affinity purified rabbit anti-chick brain tubulin antibody was a kind gift from Dr Daniel Louvard, EMBL, Heidelberg. Vinblastine sulphate, creatine kinase, theophylline and creatine phosphate were from Sigma. Cyclic AMP, ATP, GTP–Na-salt and triethanolamine–HCl were from Boehringer. Radiochemicals were from the Radiochemical Centre, Amersham. All other chemicals were of AR quality from BDH Chemicals.

3. Results

Basal adenylate cyclase activity was apparently unaffected by the addition of vinblastine sulphate up to 100 μM (fig.1). This contrasts with the situation when GTP was used to stimulate the enzyme, in which case a complex result was obtained indicating both inhibition and activation phenomena depending upon the concentration of vinblastine present.

When glucagon was added to the assay then vinblastine had a marked inhibitory effect. This inhibition could apparently be dissected into two components each of which caused 20–25% inhibition of the total activity but occurred at very different concentrations (fig.2). However, when GTP was added to the glucagon-stimulated assays then little if any inhibition was observed over the entire range of drug concentrations tested (fig.2).

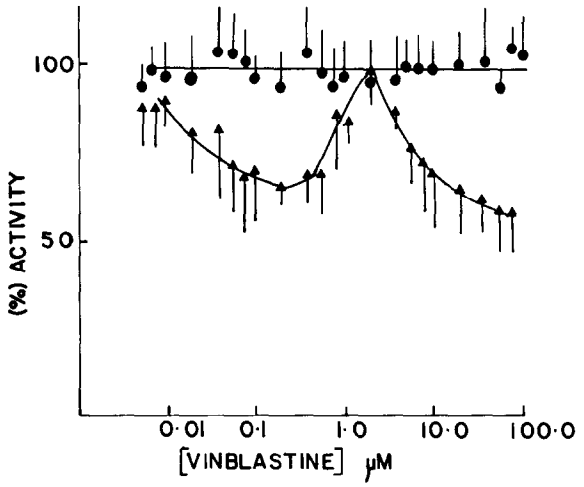

Fig.1. The effect of vinblastine on the basal and GTP-stimulated adenylate cyclase activity of liver plasma membranes. Adenylate cyclase activity of liver plasma membranes in the absence (•) and presence (▲) of 100 μM GTP. Errors are given as ±SD, n = 6 using 3 different membrane preparations. Adenylate cyclase activity was assessed from linear timecourses of cyclic AMP production over 10 min at 30°C. The assay media was at a final pH 7.4 containing final conc. 25 mM triethanolamine–HCl, 1 mM EDTA, 1 mM theophylline, 5 mM $MgSO_4$, 7.4 mg/ml creatine phosphate, 1 mg/ml creatine phosphate and 1.5 mM ATP. Cyclic AMP produced during the assay was monitored as detailed in [10]. Basal activity of adenylate cyclase was 22 μunits/mg protein, and in the presence of 100 μM GTP was 60 μunits/mg protein. (One unit is defined as 1 μmol substrate transformed/min.)

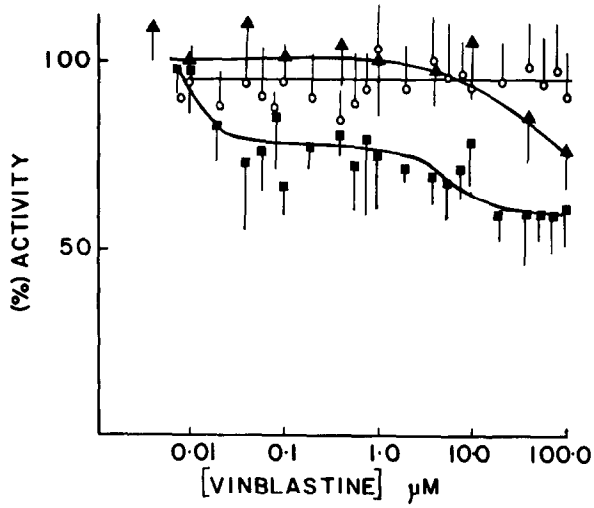

Fig.2. The effect of vinblastine on ^{125}I-specific glucagon binding to plasma membranes and on the glucagon-stimulated adenylate cyclase activity. ^{125}I-Specific glucagon binding (▲) and glucagon-stimulated adenylate cyclase activity in the absence (■) and presence (○) of 100 μM GTP. Errors are given as ±SD, n = 6 using 3 different plasma membrane preparations. Adenylate cyclase was assayed as in fig.1. The specific activity of adenylate cyclase at 30°C in the presence of 10^{-6} M glucagon was 660 μunits/mg and in the presence of glucagon plus 100 μM GTP was 1.22 munits/mg. Specific binding of ^{125}I-labelled glucagon was carried out in a buffer of final pH 7.4 containing 15 mM triethanolamine–HCl, 1 mM EDTA, 1 mM theophylline, 5 mM $MgSO_4$ and 2.5% bovine serum albumin with 10^{-9} M ^{125}I-labelled glucagon [11] for 10 min at 30°C. The specific component was assessed by adding 10^{-6} M unlabelled glucagon (see [11]). Specific binding of ^{125}I-labelled glucagon was 2.0 pmol/mg protein.

When the ^{125}I-specific glucagon binding was followed we observed a loss of ~25% of the binding at the higher vinblastine concentrations (fig.2). This phenomenon occurred at similar vinblastine concentrations to the second phase of inhibition of the glucagon-stimulated activity (fig.2).

These changes in activity were found to be effectively instantaneous (<1 min) as time courses for the assays were linear over a 10 min period and not reversed by a simple washing procedure.

Arrhenius plots of glucagon-stimulated adenylate cyclase activity were carried out in the presence and absence of 40 μM vinblastine sulphate using 3 different membrane preparations (errors are ±SD). In the absence of vinblastine a break point at 27.5 ± 0.7°C was obtained with activation energies of 36 ± 5 and 76 ± 10 kJ . mol^{-1} above and below the break point, respectively. In the presence of vinblastine a break point at 27.5 ± 2.3°C was obtained with activation energies of 28 ± 4 and 55 ± 7 kJ . mol^{-1} above and below the break, respectively.

Treatment of membranes with an affinity purified rabbit anti-chick brain tubulin antibody had no effect on basal or any of the ligand-stimulated activities.

4. Discussion

Vinblastine has profoundly different effects on the various ligand-stimulated activities of liver plasma membrane adenylate cyclase. The basal activity of the enzyme was apparently unaffected at all concentrations of vinblastine tested implying the insensitivity of the catalytic unit of the enzyme to this agent. This was not true for the GTP-stimulated activity which

exhibited marked changes in activity in response to vinblastine (fig.1). There appeared to be 2 or 3 component effects of vinblastine on the GTP-stimulated activity of the enzyme. These were an inhibitory phenomenon occurring at low vinblastine concentrations (0.01–0.5 μM) followed by a phase of activation at ~1 μM vinblastine which sufficed to relieve this inhibition. Increases in vinblastine to >2 μM led to a further phase of inhibition of activity which appeared to plateau out at ~50% inhibition. This GTP-stimulated activity is believed to involve a regulatory protein associated with the catalytic unit [8,13,14]. It is presumably at this regulatory site that vinblastine acts to cause the marked changes in activity noted. That these phenomena are only observed with the GTP-stimulated state (in the absence of hormone) suggests that they are due to vinblastine affecting either the guanine nucleotide regulatory unit associated with the catalytic unit of adenylate cyclase or it may also perturb the mechanism by which this regulatory unit activates the catalytic unit, or the catalytic unit activated in such a fashion by GTP may become sensitive to vinblastine action. This is the only stimulated state of the enzyme that exhibits an apparent activation by vinblastine (seen as a relief of an inhibition) emphasising the difference of this state from other stimulated states of the enzyme. The low concentrations of vinblastine necessary to elicit the activation and inhibitory effect might suggest that there are specific sites on this GTP binding protein that bear analogy with tubulin [1–4]. As GTP attains ~0.6 mM in the cell [15] it is likely that this state would be physiologically relevant.

The inhibition by vinblastine of the glucagon-stimulated state could seemingly be dissected into two components. The loss of activity at the higher vinblastine concentrations being due to an apparent loss of receptors as indicated by a proportional decrease in ^{125}I-labelled glucagon specific binding. This would be predicted by the mobile receptor model which is believed to apply in this instance [11,16], where one occupied receptor can activate but a single catalytic unit and there is no large excess of receptors over catalytic units [11,16].

The inhibition at the lower concentrations was presumably due to a direct action of vinblastine on the coupled transmembrane complex of receptor and catalytic unit that would be formed in the presence of glucagon [11,16]. Such a state appears to be dependent on acidic phospholipids [17] and is peculiarly sensitive to inhibition by positively charged anaesthetics [18] and lysolecithins [19]. It is not unlikely that a compound that can interact strongly with membranes and acidic species [1,3,20] by virtue of its cationic nature should effect such an inhibition. It is then very interesting that the vinblastine inhibited states of glucagon-stimulated adenylate cyclase should be abolished by the addition of GTP to the assays. In the presence of glucagon, GTP binds to a further regulatory component which becomes involved in the coupling process between the receptor and catalytic unit [8,14]. This leads to a fundamental change in mechanism, where instead of a transmembrane complex of receptor and catalytic unit being formed upon hormone addition [11], a free, activated catalytic unit is produced after interaction with the receptor and regulatory coupling component [16,21]. The free-activated form of the catalytic unit produced by addition of GTP and glucagon is in a very different state from the much less active catalytic unit formed in the presence of GTP alone [16,21] and thus would not be expected to respond similarly to vinblastine. A further consequence of this change in mechanism is that in the presence of GTP an occupied receptor can activate more than one catalytic unit: probably about 4 [16]. If, as seems likely, the inhibition of the glucagon-stimulated activity at the higher vinblastine concentrations was due to the inhibition of glucagon binding and thus the loss of occupied receptors, it is not perhaps too surprising that in the presence of GTP the inhibition of the glucagon-stimulated rate was reversed. Thus the GTP + glucagon stimulated activity is relatively insensitive to receptor losses and certainly of the amount experienced in this instance (see [16]).

In the presence of GTP and glucagon then no complex between the receptor and catalytic unit will occur as an accumulating active species. Vinblastine would not then be expected to cause an inhibition at the lower concentrations if such an inhibition was indeed due to a peculiar sensitivity of the transmembrane complex to vinblastine. This change in sensitivity may be due to an altered conformational state of the enzyme as suggested [16,21]. Alternatively the change in the disposition of the catalytic unit within the bilayer when it is not physically associated with the receptor [22] may lead to the loss of any perturbation vinblastine might achieve between the headgroups of acidic phospholipids and the catalytic unit.

Vinblastine sulphate has been shown to affect

Na^+/K^+ ATPase and other membrane transport systems [23,24] and it has been suggested that this may be due to a general membrane expansion effect [20]. This, however, is unlikely to account for the inhibitory or activation phenomena observed in this study as the concentrations of vinblastine necessary to achieve membrane expansion [20] are some orders of magnitude higher than those tested here. Arrhenius plots of glucagon-stimulated adenylate cyclase activity demonstrated a well-defined break at ~28°C due to a lipid-phase separation occurring in the membrane [10]. This was unaffected by the presence of vinblastine which mitigates against any gross perturbation of the lipid phase mediating these phenomena.

It has been suggested that the organisation of membrane proteins may be regulated by microtubule assemblies that interact with the plasma membrane and that these would be disrupted by vinblastine [25,26]. If tubulin was associated with any of the protein species of our system and this was responsible for the effects of vinblastine then crosslinking of such components using anti-tubulin antibody might well be expected to alter the response to those ligands where free lateral diffusion of components is a prerequisite for activation [11,16,21]. However, in our hands anti-tubulin antibody had no effect on any ligand-stimulated activity.

The simplest interpretation of our results is that vinblastine can act directly on the various components of the adenylate cyclase system. These sites can be identified by the susceptibility of different ligand-stimulated activities to inhibition. We propose that there are two sites affecting the action of glucagon on the adenylate cyclase system. These are a site of high affinity which perturbs the coupling between the receptor and catalytic unit of adenylate cyclase, occupancy of which causes inhibition. A further site of lower affinity inhibits binding of glucagon to its receptor, which again causes loss of hormone-stimulated activity. There then appears to be 2 or 3 other sites that are apparent from observations of the GTP-stimulated state (in the absence of hormone) and are presumably associated with the GTP-regulatory unit attached to the catalytic unit. Occupancy by vinblastine of these sites can lead to either inhibition or activation phenomena. That such changes occur in the activity of adenylate cyclase to vinblastine, and at such low concentrations, requires that care be taken when attributing the mode of action of vinblastine on cellular systems. This is especially true when many phenomena are affected by cyclic nucleotide levels and the vinblastine concentrations used to disrupt microtubules are in the range affecting adenylate cyclase activity. Our results may have significance in the use of vinblastine for treating Hodgkin's disease and certain cancers [27]. For at the doses used in clinical practice it is not unlikely that blood levels may attain 0.05–0.1 μM vinblastine [27], which would suffice to allow the occurrence of some of the effects discussed here.

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