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Decreased selectivity of vasoactive intestinal peptide receptors by GTP

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High-affinity receptors for vasoactive intestinal peptide (VIP) bind peptides structurally homologous to VIP with relatively low affinity [1–3]. These peptides include growth hormone releasing factor (GRF), peptide histidine isoleucine (PHI) and secretin. Guanosine triphosphate (GTP) and its analogs inhibit VIP-receptor binding and potentiate cyclic AMP synthesis in response to VIP [1, 4, 5], suggesting a role for GTP-binding regulatory protein(s) (G-protein) in VIP-receptor regulation. We observed previously that detergent-induced disaggregation of a GTP-sensitive, VIP-preferring receptor complex solubilized from guinea pig lung membranes produces a binding species that is GTP insensitive and GRF preferring [6]. This observation suggested that a G-protein may influence the binding selectivity of VIP receptors. However, detergents interact intimately with integral membrane proteins [7] and may alter the binding characteristics of receptors. To eliminate the possible confounding influence of the detergent, we have measured the effect of GTP on the binding selectivity of VIP receptors present in intact lung membranes.

Materials and Methods

[^{125}I]*VIP*. Pure porcine VIP (provided by Dr. S. I. Said, Illinois University, College of Medicine) was labeled with [^{125}I] and purified according to Ref. 8, except that the iodination was for 30 sec with 25 μg chloramine-T, and the purification of [^{125}I]VIP was by reverse phase HPLC on a single Novapak-C18 column (Waters, Milford, MA) to a specific activity of 2000 Ci/mmol [8], a value close to the maximum specific activity of monoiodinated [^{125}I]VIP. This preparation was used routinely as tracer in the receptor binding studies. For comparison, some binding assays were also performed using [(^{125}I)-Tyr^{10}]VIP purchased from Amersham, Arlington Heights, IL.

Lung membranes. Freshly dissected guinea pig lungs were perfused (8 ml/min) with about 75 ml Krebs buffer, pH 7.4, until the tissue became near-white in color [9]. The lungs were homogenized twice (30 sec, 10,000 rpm; Biohomogenizer model 133/1281-0 Biospec Products, Bartlesville, OK) on ice in 5 vol. of 10 mM Trizma–HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA (sodium salt), 100 units aprotinin/ml, 100 μM phenylmethylsulfonyl fluoride, and 5 μM pepstatin A (Sigma Chemical Co., St. Louis, MO). The homogenate was strained through six layers of medical gauze and centrifuged (30,000 *g*, 30 min, 4°), and the pellet was resuspended to 10–15 mg protein/ml in homogenization buffer, assayed according to Ref. 10.

[^{125}I]*VIP receptor binding*. The membranes (50 μg protein in 50 μl) were incubated (30 min, 23°) with [^{125}I]VIP (100 μl, 60–80 pM) in the absence and presence of increasing concentrations of unlabeled porcine VIP or rat GRF(1–44) (50 μl; Peninsula Laboratories, Belmont, CA) in polypropylene microfuge tubes previously treated (10 min) with 0.5 ml of assay buffer (100 mM Trizma–HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1% bovine serum albumin). Termination was by addition of 0.5 ml of assay buffer, centrifugation (12,000 *g*, 3 min), and aspiration of the supernatant fractions. Radioactivity in the pellets was determined by gamma spectrometry (Beckman, model 5500). Saturable binding (a term synonymous with "specific binding" in this paper) was the radioactivity displaced by a 1 μM concentration of unlabeled VIP. Time course experiments (not shown) indicated that steady-state saturable binding of [^{125}I]VIP was achieved under these conditions. To interfere with G-protein : receptor coupling, GTP or its nonhydrolyzable analog, guanyl-5′-yl imidodiphosphate (GppNHp) (Sigma), was included in the assay mixture. K_d and B_{max} for VIP and GRF were computed using the computer programs EBDA and LIGAND (Elsevier Biosoft, Cambridge, U.K.) run on an IBM-AT computer [11]. K_d for [^{125}I]VIP, required for computation of K_d for GRF, was determined by assaying saturable binding at increasing [^{125}I]VIP concentrations (5–500 pM). Hill slopes close to unity (0.95–1.002) and linear Scatchard plots suggested that the binding of [^{125}I]VIP was predominantly by a single population of receptors with K_d values of 0.38

and 1.05 nM for untreated and GTP-treated membranes respectively. The coefficients of variation for K_d and maximum binding capacity values were usually 5–10%, and never >17%. Receptor selectivity index (SI) was computed as the ratio of IC_{50} or K_d values for GRF and VIP.

Results and Discussion

Increasing concentrations of GTP caused progressive decreases in saturable [^{125}I]VIP binding by guinea pig lung membranes (Fig. 1). Gpp(NH)p, a nonhydrolyzable GTP analog, inhibited the binding with a potency nearly equivalent to that of GTP, whereas ATP was without effect on the binding. The potency of unlabeled VIP in displacing [^{125}I]VIP binding by the untreated membranes was 26.3- and 72.1-fold greater than those of the homologous peptides GRF (Fig. 2A) and PHI (not shown), indicating that the binding was to VIP-preferring receptors. Treatment with GTP (0.1 mM) increased the IC_{50} values (concentrations giving 50% inhibition of saturable binding) for VIP as well as GRF, but the increase was greater for VIP than for GRF (Fig. 2 and Table 1). Thus, the receptor selectivity (IC_{50} for GRF/IC_{50} for VIP) was reduced by the GTP treatment (by 4.6-fold). The reduced selectivity of VIP binding appeared to arise from an action of GTP on the high-affinity receptor population. This conclusion was suggested by computer-assisted analysis [11] of K_d values for VIP and GRF binding by untreated and GTP-treated membranes. The displacement curves were consistent with the presence of two VIP receptor populations in untreated membranes with K_d values of 0.3 and 77 nM, and binding capacity (B_{max}) of 0.6 and 4.4 pmol VIP/mg protein respectively (N = 3). GTP treatment increased the K_d for the high-affinity receptor population (by 5.3-fold) but did not influence the K_d of the low-affinity receptor population or the binding capacities of either type of receptors. The GRF-displacement data suggested a single binding site with a K_d of 13.9 nM and a B_{max} of 0.92 pmol GRF/mg protein. GTP treatment increased the K_d for GRF (Table 1), without influencing the GRF-binding capacity. Receptor selectivity, computed as the ratio of K_d values for GRF and VIP, was reduced by 3.3-fold in the GTP-treated membranes.

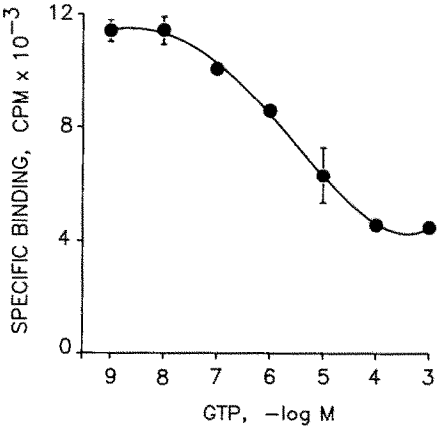

Fig. 1. Decreased [^{125}I]VIP binding by lung membranes treated with GTP. Values, means of three replicates (± SD), represent saturable binding displaced by a 1 μM concentration of unlabeled VIP. The binding measured in the absence of GTP was 11,500 ± 678 cpm.

These observations suggest that GTP caused a decrease in receptor affinity for VIP that was larger than the decrease in affinity for GRF. This effect is likely to arise from a GTP-mediated interference with receptor : G-protein coupling in the membranes. This conclusion is consistent with our previous observation that detergent-induced conversion of a VIP-preferring receptor complex to a GRF-preferring binding species is accompanied by a loss of GTP sensitivity of the binding [6]. GRF is a partial agonist for VIP receptors [12]. The results of the present study are consistent with findings [13] that guanyl nucleotides influence the binding of a full-agonist by β-adrenergic receptors more than that of partial agonists. These considerations suggest that receptor : G-protein coupling is a likely contributory factor in the selective binding of VIP by the lung.

A heterologous peptide-receptor binding system composed of porcine VIP, rat GRF and guinea pig receptors was employed in this study. The primary structures of VIP and GRF appear to be fairly well-conserved in mammals [14], and VIP receptors present in guinea pig and other mammals exhibit similar binding affinities and sensitivities to guanyl nucleotides [1, 2, 6]. Thus, the G-protein modulation of VIP receptor selectivity observed in this study is likely to occur in systems where VIP receptors and peptide ligands originate from the same species. Another relevant concern is the homogeneity of the [^{125}I]VIP employed as the tracer (see Ref. 15 for discussion) and the possibility that different forms of the tracer (e.g. [(^{125}I)-Tyr^{10}]VIP and [(^{125}I)-Tyr^{22}]VIP may bind different types of VIP receptors. Although the [^{125}I]VIP tracer used in this study requires further characterization with respect to position and degree of labeling with [^{125}I], a number of considerations suggest that the observed GTP effects do not derive from heterogeneity of the tracer preparation. These include: (i) the [^{125}I]VIP preparation is purified as a single peak by resolutive reverse phase HPLC [8], (ii) analysis of receptor binding measured at increasing tracer concentrations suggested a single [^{125}I]VIP binding site in the untreated (K_d: 0.38 nM) and GTP-treated membranes (K_d: 1.05 nM), and (iii) unlabeled VIP displaced the binding of [(^{125}I)-Tyr^{10}]VIP (purchased from the Amersham Corp.) by untreated and GTP-treated membranes with IC_{50} values 0.5 and 4.4 nM, respectively, values close to those obtained using our own [^{125}I]VIP preparation as the tracer (0.6 and 4.0 nM).

Table 1. Reduced VIP binding selectivity of lung membranes by GTP

	IC_{50}^{*} (nM)			K_d‡ (nM)		
	VIP	GRF	SI_a†	VIP	GRF	SI_b†
No GTP	0.6	15.8	26.3 ± 4.5	0.3	13.9	46.3 ± 9.5
+GTP (0.1 mM)	4.0	24.0	5.9 ± 2.0	1.6	24.1	14.1 ± 3.3

Values are means of three experiments each.
* Concentration giving 50% inhibition of saturable [^{125}I]VIP binding.
† Selectivity index. SI_a = (IC_{50}, GRF/IC_{50}, VIP); SI_b = (K_d, GRF/K_d, VIP); means ± SEM.
‡ Equilibrium dissociation constant.

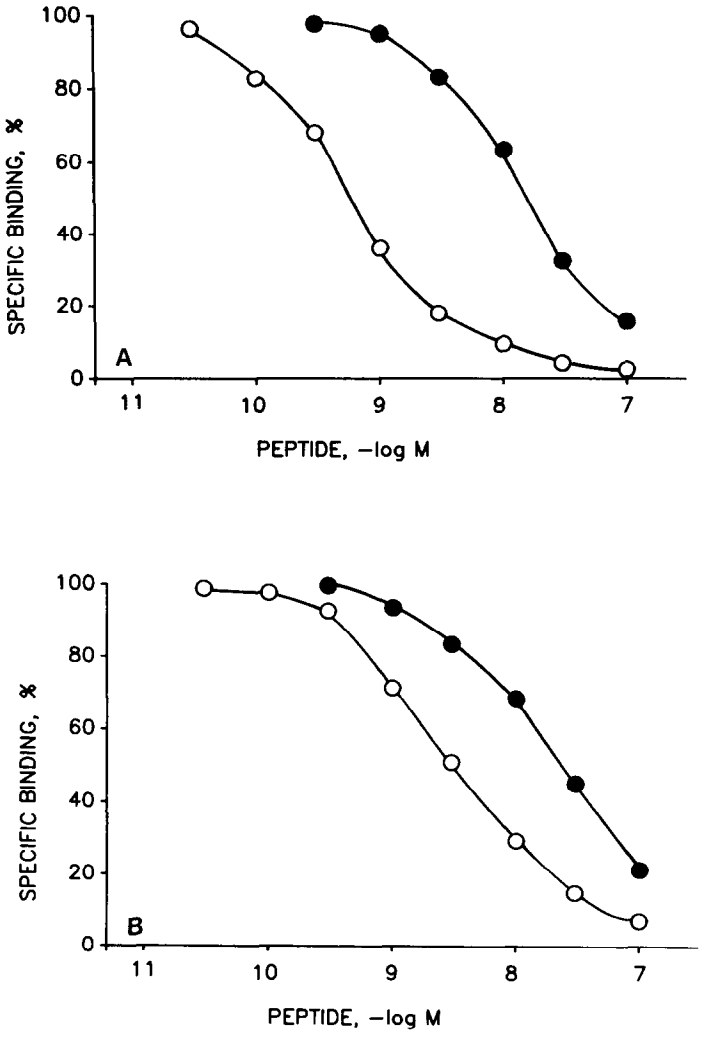

Fig. 2. Displacement of saturable [^{125}I]VIP binding to lung membranes by unlabeled VIP (○) and GRF (●). (A) and (B) show displacement curves obtained in the absence and presence of GTP (0.1 mM) respectively. Values are means of triplicates from typical experiments expressed as percent of saturable binding in the absence of unlabeled peptides [(A): 10,400 cpm, (B): 6,290 cpm]. The values for non-specific binding in (A) and (B) were 1,010 and 1,180 cpm respectively.

In conclusion, GTP increased the IC_{50} and K_d values for binding of VIP and GRF to lung membranes, but the increases were greater for VIP than for GRF. This resulted in reduced VIP-binding selectivity of the membranes (by 3.3- to 4.6-fold). Thus, a G-protein(s) is likely to influence the binding selectivity of VIP receptors present in the lung.

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A time study on the uptake of estramustine into prostatic tumour 1013L cells *in vitro*

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The nuclear protein framework, the nuclear protein matrix, NPM, has attracted much attention during the last years due to its probable role in several important biological processes [1], e.g. DNA synthesis. RNA synthesis, processing and transport and hormone action. We have earlier investigated the role of the NPM in estramustine (EM) induced cell death. In HeLa cells, highly sensitive to EM, we found a high amount of intact EM and several metabolites hydrofobically bound to the NPM [2]. This preferential binding was also found in the human prostatic tumour 1013L cell line, with an increase in NPM uptake at higher cell densities [3]. Other results have shown that EM acts as an antimitotic agent arresting cells in metaphase [4] and causing an inhibition of the assembly and a disassembly of microtubules via interaction with the microtubule-associated proteins [5, 6]. However, other data indicate that cytotoxicity is also mediated via binding to the NPM [7].

Although no indication of DNA damage has been found for EM [2], the important role of the NPM gives cause for further studies at the nuclear level. In fact, we have earlier studied the effect of EM on specific RNA labelling in 1013L cells. An inhibition of all the different RNA-species was found, indicating a mode of action involving nuclear targets [8]. From our previous experiments in 1013L cells, we also know that EM and its oxidative metabolite estromustine are retained by the NPM for a long time, 45–50% being still bound after a 7 hr drug free recovery period [7]. This is higher than that found for other anti-mitotic agents, such as vinblastine and vincristine, where after 3 hr recovery 10 and 30% respectively were found to be retained intracellularily [9]. Thus, although estramustine exhibits typical characteristics of an anti-mitotic agent, this indicates that its uptake kinetics might differ from that of the vinca-alkaloids. We therefore deemed it of interest to study the uptake kinetics of EM over an extended time period at the cellular, nuclear and NPM level.

Materials and methods

^{3}H-estramustine, estradiol 3-*N*-bis[2-chloroethyl] carbamate (2, 4, 6, 7-^{3}H; 102 Ci/mmol) was synthesized at AB Leo. The purity of the compound was at least 98% as determined by high performance liquid chromatography, HPLC (Waters Bondapak C18 column, acetonitrile: water: acetic acid (63:36:1); 0.4 ml/min). The substance, stored in 9:1 toluene: ethanol, was evaporated with N^2 (g) and dissolved in 95% ethanol. The final concentration of solvent in the incubation mixture did not exceed 0.2%.

The human prostatic 1013L tumour cell line [10] was kindly donated by Dr. D. Mickey, Duke University, U.S.A. The cells were grown as described earlier [3]. HeLa S_3 cells were grown in minimum essential medium (S-MEM) supplemented with 10% FCS. The cells were constantly stirred (40 rpm) and diluted at 10^6 cells/ml. Cells in late log phase ($0.5–0.7 \times 10^6$ cells/ml) were incubated with 10 nM ^{3}H-EM at 37° from 5 min to 24 hr. Incubations were terminated by centrifugation of the cells in a chilled centrifuge (1000 rpm) and subsequent washes with ice-cold medium without serum. Nucleus and nuclear protein matrix were isolated as described earlier [3].

For determinations of drug uptake the cellular, nuclear and NPM fractions were combusted in a Packard Sample Oxidizer and counted in 10 ml Instagel (Packard) by a liquid scintillation counter for 10 min. Protein measurements were carried out according to Lowry [11].

Results and discussion

Figure 1 shows the cellular, nuclear and NPM uptake of ^{3}H-EM in 1013L cells. After 1 hr, approximately 30% of the final binding was found associated with the nuclear and NPM fractions, but the maximum was not reached until 15 to 20 hr from the start of the incubation. ^{3}H-EM binding to whole cells was, however, a faster process, after 5 min