British Journal of Pharmacology (1996) 117, 111-118

Functional properties of the uptake of amines in immortalised peptidergic neurones (transport-P)

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1 Most neurotransmitters are inactivated by uptake into presynaptic nerve terminals and into glial cells. We recently provided evidence for uptake of amines in postsynaptic neurones. Uptake was evident at nanomolar concentrations of prazosin, but at concentrations of unlabelled prazosin greater than 10^{-7} M, there was a further activation of uptake, manifested by a paradoxical increase in accumulation of the radioligand. We have now studied further characteristics of amine uptake in immortalised gonadotrophin-releasing hormone (GnRH) neurones. Control cells included SK-N-SH neuroblastoma cells (which possess presynaptic type amine transporters) and non-neuronal (COS-7) cells.

2 [^{3}H]-prazosin bound to intact GnRH cells and was displaced by unlabelled prazosin in concentrations of 10^{-9} to 10^{-7} M. However, at higher concentrations of unlabelled prazosin, there was an increase in apparent [^{3}H]-prazosin binding, as we had previously described. This paradoxical increase in accumulation of the radioligand was abolished by desipramine.

3 Desipramine had no effect on the association of prazosin with COS-7 cells. There was no paradoxical increase in accumulation of [^{3}H]-prazosin in COS-7 cells, indicating that this effect requires the presence of a desipramine-blockable uptake process.

4 The increase in binding of the radioligand that was observed in the GnRH cells is not a general property of neuronal transporters; in SK-N-SH cells, there was no increase in accumulation of (−)-[^{3}H]-noradrenaline in the presence of concentrations of unlabelled (−)-noradrenaline greater than 10^{-7} M.

5 The uptake of prazosin and the increase in accumulation of [^{3}H]-prazosin were abolished in the cold, indicating that this is an active, energy-requiring process.

6 Desipramine-sensitive uptake of prazosin was demonstrable in the GnRH cells in the absence of sodium. Further, the Na^+/K^+-ATPase inhibitor, vanadate, abolished noradrenaline uptake in SK-N-SH cells but had no effect on prazosin uptake in GnRH cells. Thus, the uptake of prazosin does not derive its energy from the sodium pump.

7 Prazosin uptake was inhibited by the V-ATPase inhibitor bafilomycin A_1, the H^+/Na^+ ionophore, monensin and the organic base, chloroquine, indicating that uptake derives its energy from a proton pump. In contrast to other proton-dependent amine transporters, the uptake of prazosin was unaffected by reserpine.

8 Increasing extracellular pH did not increase the uptake of prazosin into GnRH cells, indicating that it is unlikely to be due to non-specific diffusion and concentration of a lysosomotropic drug into intracellular acidic particles.

9 The uptake of prazosin was unaffected by steroid hormones.

10 In COS-7 cells transfected with α_1-adrenoceptor cDNA, [^{3}H]-prazosin was displaced by unlabelled prazosin without causing an increase in binding of the radioligand. This indicated that the increase in accumulation of the radioligand is unlikely to be due simply to some function of α_1-adrenoceptors.

11 Thus, peptidergic neurones possess an uptake process with properties that are distinguishable from known amine transporters.

Keywords: Amine uptake; hypothalamus; prazosin; neurones; biological transport

Introduction

The peptidergic neurones of the hypothalamus are densely innervated by noradrenergic nerve terminals and noradrenaline plays an important role in regulating the neuroendocrine functions of the hypothalamus. Activation of α_1-adrenoceptors which are located on the peptidergic neurones ultimately stimulates the secretion of several pituitary hormones, including adrenocorticotrophin, thyrotrophin, prolactin and the gonadotrophins. Some of these effects have been shown to be important in the physiological control of secretion of these hormones in man and in rats (for a recent review, see Al-Damluji, 1993).

While studying the functional properties of α_1-adrenoceptors in the hypothalamus, we observed a high-affinity uptake process for amines in peptidergic neurones (Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al.*, 1993). In primary hypothalamic cell cultures and in a hypothalamic neuronal cell line, [^{3}H]-prazosin bound with high affinity and was displaced by unlabelled prazosin in concentrations of 10^{-10} to 10^{-7} M. However, at concentrations of unlabelled prazosin greater than 10^{-7} M, there was a paradoxical increase in apparent [^{3}H]-prazosin binding, which could be abolished by desipramine; in the presence of desipramine, unlabelled prazosin displaced [^{3}H]-prazosin as before, but no increase in accumulation was seen above 10^{-7} M. The paradoxical increase of [^{3}H]-prazosin binding was not observed in membrane preparations of hypothalamic neurones. We interpreted these findings as indicating the presence in the hypothalamic neurones of α_1-

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adrenoceptors and a prazosin uptake process. Desipramine-blockable uptake was evident at nanomolar concentrations of prazosin. As the concentration of unlabelled prazosin is increased, [^{3}H]-prazosin is displaced from the receptors. The cellular uptake process becomes activated at higher concentrations of the ligand. This activation is manifested by a paradoxical increase of the amount of radioligand in the cells, despite the fall in specific activity of [^{3}H]-prazosin, consequent upon dilution with unlabelled prazosin (Al-Damluji *et al.*, 1993). Small amounts of noradrenaline were also taken up with high affinity by both types of hypothalamic cell cultures. Studies in rats suggested that the postsynaptic uptake process is operative in hypothalamic corticotrophin-releasing hormone and vasopressin neurones *in vivo* (Al-Damluji *et al.*, 1993). We suggested that the physiological function of the postsynaptic uptake process may be to remove neurotransmitter from the vicinity of postsynaptic receptors, thus preventing desensitization of the postsynaptic neurones and maintaining their responsiveness to repeated bursts of neurotransmitter released from the presynaptic nerve terminals. In contrast, the presynaptic uptake processes would presumably be less effective in removing neurotransmitter from postsynaptic receptors, as they would rely on diffusion of molecules of neurotransmitter back across the synapse, against its concentration gradient (Al-Damluji *et al.*, 1993).

We have now studied in greater detail the uptake of amines in immortalised gonadotrophin-releasing hormone (GnRH) neurones. These cells retain many of the properties of differentiated hypothalamic neurones, such as extension of dendrites, expression of neuronal cell markers, spontaneous electrical activity via ion channels and storage, processing and release of the neurotransmitter GnRH in a pulsatile manner which is characteristic of its secretion *in vivo* (Mellon *et al.*, 1990; Liposits *et al.*, 1991; Krsmanovic *et al.*, 1992; Weiner *et al.*, 1992; Wetsel *et al.*, 1992). Our findings indicate that the uptake of amines in these cells is via a process with properties that are distinct from known transporters.

Methods

Cell culture

Immortalised GnRH neuronal cells (GT1-1 cells; Mellon *et al.*, 1990) were generously provided by Dr R.I. Weiner. SK-N-SH neuroblastoma cells (Biedler *et al.*, 1973) were obtained from the American Type Culture Collection (ATCC catalogue number HTB-11; batch number F-10164), as were COS-7 cells (Gluzman, 1981; ATCC catalogue number CRL-1651; batch number F-10556).

The cells were grown in Corning 75 cm^2 flasks in culture medium consisting of DMEM and F-12 (ratio 1 : 1) containing 10% heat-inactivated foetal bovine serum, sodium bicarbonate 3.7 g l^{-1} and gentamicin 100 mg l^{-1}. When the cells reached confluence, they were dispersed by incubation for 10 min at 37°C with phosphate-buffered saline (PBS) containing 0.05% trypsin, 0.05% EDTA, 0.05% $NaHCO_3$, 0.02% DNAse I, 0.1% glucose and 0.01% gentamicin. The cell suspension was centrifuged for 10 min at 400 *g* and the supernatant discarded. The cells were washed once in culture medium and were then re-suspended in fresh medium at a density of 10^6 cells/ml and incubated in Costar 12-well plates (2×10^6 cells/well). Culture media were changed at 48 h intervals and the experiments were carried out four days following culture.

Transfection of COS-7 cells

COS-7 cells were dispersed in culture medium (DMEM + F-12 + 10% foetal bovine serum) at a density of 0.1×10^6 cells ml^{-1} and incubated in Falcon 6-well plates, 2 ml per well (0.2×10^6 cells per well; 22,000 cells cm^{-2}). Twenty four hours later, when the cells were 60–70% confluent, they were washed with 2 ml of Opti-MEM serum-free medium and then exposed for 24 h to a mixture of α_{1B}-cDNA (1 μg) and cationic liposomes (Lipofectamine, 12 μl) in serum-free medium. The mixture had been allowed to equilibrate at room temperature for 45 min to allow DNA-liposome complex formation, prior to addition to the cells. The cells were incubated with the complexes at 37°C in an atmosphere of 5% CO_2 for 24 h, after which the complexes were removed and repltaced with fresh serum-containing medium. Forty eight hours after the start of exposure to the DNA-liposome complexes, the intact cells were analysed for accumulation of [^{3}H]-prazosin as described below.

Uptake and binding studies

Uptake studies were performed at 37°C (unless otherwise indicated) on the intact cells. For experiments in the cold, the culture wells were placed on ice. Drugs were dissolved in 'binding medium' consisting of DMEM with 25 mM HEPES and 0.5×10^{-3} M sodium ascorbate, pH 7.4. In the experiment which examined the effect of extracellular alkalinity, small amounts of 1 M Tris base were added to the 'binding medium' to obtain a series of solutions with pH varying from 7.40 to 8.10 by increments of 0.10 units.

For experiments that examined the role of sodium, the uptake buffer ('Krebs-Ringer-HEPES') consisted of (mM): sodium chloride 125.0, potassium acetate 5.0, HEPES 25.0, magnesium sulphate 1.2, potassium phosphate 1.2, glucose 10.0, ascorbic acid 1.0 and calcium lactate $5H_2O$ 2.5. The pH was adjusted to 7.4 with Tris base. For experiments in the absence of sodium , sodium chloride was replaced with Tris HCl 125 mM. In the experiments which examined the effect of vanadate, potassium phosphate was excluded from the 'Krebs-Ringer-HEPES' buffer to enhance entry of vanadate into the cells (Cantley *et al.*, 1978).

The concentration of the radioligand was $2-3 \times 10^{-9}$ M and unlabelled drugs were added at the concentrations indicated. The cells were washed twice with 1 ml of the appropriate uptake buffer and then incubated in buffer containing the labelled ligand, alone or in combination with unlabelled drugs. At the end of the incubation period, the wells were placed on ice and the cells were washed twice with 1 ml volumes of ice-cold buffer. The cells were then solubilized with 2 ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M sodium hydroxide. Fifty μl aliquots were removed for protein assay and 10 ml of scintillation liquid was then added to the cell extract, mixed and radioactivity was measured in a beta scintillation counter with an efficiency of 50%. Protein content was measured by the bicinchoninic acid modification of the biuret reaction (Smith *et al.*, 1985) using albumin standards and reagents supplied by Pierce (Rockford, Illinois, U.S.A.).

Materials

[^{3}H]-prazosin was obtained from Amersham (TRK.843; specific activity 74–83 Ci mmol^{-1}; batch numbers 28, 29, 31–33). (−)-[7,8−^{3}H]-noradrenaline was also from Amersham (TRK.584; specific activity 42–43 Ci mmol^{-1}; 37% of ^{3}H in position 7 and 63% in position 8; batch numbers 145, 146 and 151). Gentamicin and foetal bovine serum were from Gibco and culture media (DMEM and F-12) were from Sigma. Opti-MEM serum-free medium (BRL 31985) and Lipofectamine (BRL 18324-012) were from Life Technologies (Gaithersburg, Maryland, U.S.A.). α_{1B}-cDNA in pCMV was a gift from Dr R.J. Lefkowitz. All other unlabelled drugs were obtained from Sigma or Aldrich. Scintillation liquid was Hydrofluor (National Diagnostics, Manville, N.J.). The data are expressed as the means ± s.e.mean; s.e.mean bars are not shown where they are smaller than the sizes of the symbols. Each experiment has been replicated at least once with similar results.

Results

[^{3}H]-prazosin 2×10^{-9} M bound to the GnRH cells and apparent equilibrium was reached in approximately 30 min. In the presence of 10^{-6} M unlabelled prazosin, more [^{3}H]-prazosin was associated with GnRH cells (accumulation of [^{3}H]-prazosin at 120 min: control: 24,484 ± 552 d.p.m.; unlabelled prazosin 10^{-6} M: 88,138 ± 1,682 d.p.m.). Further time course studies showed that desipramine 10^{-5} M inhibited the association of prazosin 10^{-6} M with the GnRH cells (14,404 ± 414 d.p.m. at 120 min).

Comparison of GnRH cells and COS-7 cells

In GnRH cells, accumulation of prazosin 10^{-6} M at equilibrium (60 min) was inhibited by desipramine in a dose-dependent manner (IC_{50} 1.36×10^{-6} M; Figure 1). Accumulation of prazosin 10^{-6} M into COS-7 cells was much smaller than in GnRH cells (83.5 ± 2.0 vs 269.7 ± 2.5 pmol mg^{-1} protein) and desipramine had no effect on the accumulation of prazosin in the COS-7 cells (Figure 1). In the GnRH cells at equilibrium, [^{3}H]-prazosin 2×10^{-9} M was displaced by unlabelled prazosin in the concentration-range 10^{-9} to 10^{-7} M, and the paradoxical increase was evident above this concentration of unlabelled prazosin, as previously described (Al-Damluji *et al.*, 1993). In the presence of desipramine 10^{-5} M only displacement of [^{3}H]-prazosin by unlabelled prazosin was seen. There was no increase in accumulation of the radioligand in COS-7 cells which were studied in an identical manner (Figure 1).

At 2×10^{-9} M [^{3}H]-prazosin, the components of labelling in GnRH cells at equilibrium were: 31% desipramine-blockable uptake (11,266 d.p.m.), 25% receptor binding (9,236 d.p.m. displaceable by unlabelled prazosin 10^{-6} M) and 44% non-specific binding (15,998 d.p.m. remaining in the presence of desipramine and unlabelled prazosin; Figure 1b panel). In the presence of unlabelled prazosin 10^{-6} M, desipramine-blockable uptake represented 83% of the total accumulation of prazosin in the cells (Figure 1a).

Comparison of the binding of prazosin to GnRH cells with the binding of noradrenaline to SK-N-SH neuroblastoma cells

SK-N-SH cells took up (−)-noradrenaline 10^{-6} M and apparent equilibrium was reached by approximately 60 min. The uptake of (−)-noradrenaline in these cells was inhibited by desipramine in a concentration-dependent manner with an IC_{50} of 1.7×10^{-8} M (data not shown).

In SK-N-SH cells, unlabelled (−)-noradrenaline in concentrations up to 10^{-6} M had no effect on the uptake of [^{3}H]-(−)-noradrenaline at equilibrium (Figure 2). Higher concentrations of unlabelled (−)-noradrenaline reduced the amount of [^{3}H]-(−)-noradrenaline that was associated with the SK-N-SH cells, presumably due to saturation of the uptake$_1$ noradrenaline transporter (Figure 2). In contrast, in the GnRH cells which were studied in an identical manner, [^{3}H]-prazosin was displaced by unlabelled prazosin in the concentration range 10^{-8} to 10^{-7} M, and the paradoxical increase was evident above this concentration of unlabelled prazosin (Figure 2).

Energy requirements

Effect of cooling on the uptake of prazosin in the GnRH cells Cooling the GnRH cells reduced the total binding of [^{3}H]-prazosin (2×10^{-9} M) (B_0: 37°C: 38,508 ± 800 d.p.m.; 0°C: 25,974 ± 1,190 d.p.m.) but displacement of [^{3}H]-prazosin by unlabelled prazosin was still evident (Figure 2). Cooling the cells inhibited almost completely the paradoxical increase in accumulation of the radioligand at concentrations of unlabelled prazosin greater than 10^{-7} M (Figure 2).

The role of sodium in amine uptake In the SK-N-SH cells, exclusion of sodium from the extracellular space inhibited the uptake of (−)-noradrenaline 10^{-6} M as effectively as desipramine 10^{-5} M (Figure 3). At 60 min, desipramine inhibited the uptake of (−)-noradrenaline by 85% and exclusion of sodium inhibited uptake by 82% (control: 47.2 ± 0.6; desipramine: 7.0 ± 0.3; sodium absent: 8.3 ± 0.1 pmol mg^{-1} protein; Figure 3). In contrast, in the GnRH cells studied in an identical manner, exclusion of sodium caused only 30% inhibition of prazosin uptake; the uptake of prazosin that was observed in the GnRH cells in the absence of sodium could be inhibited by desipramine (Figure 3; uptake of prazosin at 60 min: control:

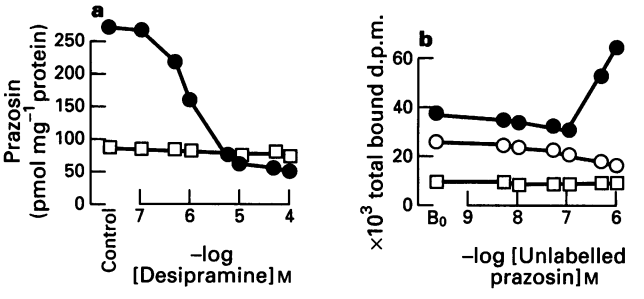

Figure 1 (a) Comparison of the effects of desipramine on the incorporation of prazosin 10^{-6} M in GT1-1 GnRH cells (●) and in a non-neuronal cell line (COS-7 cells) (□). Desipramine inhibited the incorporation of prazosin in the GnRH cells (IC_{50} 1.36×10^{-6} M) but had no effect in the non-neuronal cells. (b) Comparison of the effect of unlabelled prazosin on the incorporation of [^{3}H]-prazosin in GT1-1 GnRH cells (●) and in COS-7 cells (□). In the GnRH cells [^{3}H]-prazosin was displaced by unlabelled prazosin in concentrations up to 10^{-7} M and the paradoxical increase was evident above this concentration. Desipramine 10^{-5} M reduced the accumulation of [^{3}H]-prazosin (2×10^{-9} M) and abolished the paradoxical increase of [^{3}H]-prazosin (○). In the presence of desipramine 10^{-5} M, only displacement of [^{3}H]-prazosin by unlabelled prazosin was seen. There was no paradoxical increase in the COS-7 cells, suggesting that it requires the presence of a desipramine-blockable uptake process which is present in the GnRH cells but not in the COS-7 cells (see a).

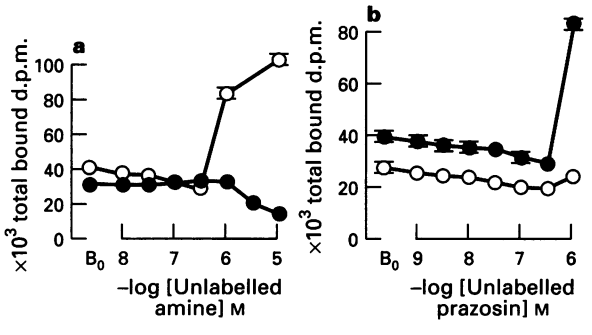

Figure 2 (a) Comparison of the binding of prazosin to GT1-1 GnRH cells (○) with the binding of (−)-noradrenaline in SK-N-SH neuroblastoma cells (●) which possess presynaptic type amine transporters. The cells were studied at equilibrium using approximately 2×10^{-9} M of the radioligand and the indicated concentrations of the unlabelled drugs. The data represent total bound d.p.m. There was no increase of the radioligand in the SK-N-SH cells; at concentrations of unlabelled noradrenaline greater than 10^{-6} M, the radioligand was displaced by the unlabelled drug, presumably due to saturation of the uptake$_1$ transporter. These comparative studies illustrate the paradoxical nature of the observations on prazosin, and indicate that the paradoxical increase is not a general property of neuronal transporters. (b) Effect of unlabelled prazosin on the incorporation of [^{3}H]-prazosin at 37°C (●) and at 0°C (○). The cells were studied at equilibrium using [^{3}H]-prazosin 2×10^{-9} M, with or without the indicated concentrations of unlabelled prazosin. The data represent total bound d.p.m.

395.4±12.1; desipramine: 61.8±1.5; sodium absent: 274.9±7.0; sodium absent+desipramine: 46.7±0.3 pmol mg^{-1} protein).

In the SK-N-SH cells, the uptake of (−)-noradrenaline was inhibited by the P-ATPase inhibitor, sodium orthovanadate (Na_3VO_4) in a concentration-dependent manner with an IC_{50} of 3.6×10^{-5} M (Figure 4). In contrast, in the GnRH cells which were studied in an identical manner, sodium orthovanadate had no consistent effect on the uptake of prazosin (Figure 4; prazosin uptake: control: 332.9±8.1 pmol mg^{-1}; vanadate 10^{-4} M: 324.4±8.9 pmol mg^{-1}). In these experiments, desipramine 10^{-5} M inhibited the uptake of amines in both the SK-N-SH and GnRH cells, confirming that active amine uptake was taking place (Figure 4). In these experiments, the entry of vanadate into the cells was facilitated by exclusion of phosphate from the Krebs-Ringer-HEPES buffer (Cantley *et al.*, 1978). In another experiment, sodium metavanadate ($NaVO_3$) inhibited the uptake of (−)-noradrenaline in the SK-N-SH cells in a concentration-dependent manner with an IC_{50} of 4.5×10^{-5} M (data not shown). Sodium metavanadate had no effect on the uptake of prazosin in the GnRH cells which were studied in an identical manner. In both of these experiments, the uptake of amines was inhibited by desipramine 10^{-5} M (data not shown).

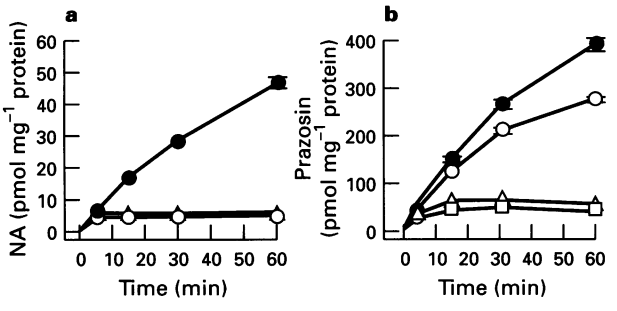

Figure 3 Effects of desipramine and exclusion of sodium on the uptake of amines in SK-N-SH neuroblastoma cells (a) and in GT1-1 GnRH cells (b). In the neuroblastoma cells (a) exclusion of sodium (○) from the extracellular space abolished the uptake of (−)-noradrenaline 10^{-6} M in a similar manner to desipramine 10^{-5} M (△); (●) control. In contrast, in the GnRH cells (b) which were studied in an identical manner, exclusion of sodium (○) had only a minor effect on the uptake of prazosin 10^{-6} M; (●) control; (△) desipramine 10^{-5} M. In the absence of sodium, there is a substantial amount of desipramine-blockable prazosin uptake in the GnRH cells (□), indicating that uptake in these cells is not dependent on sodium.

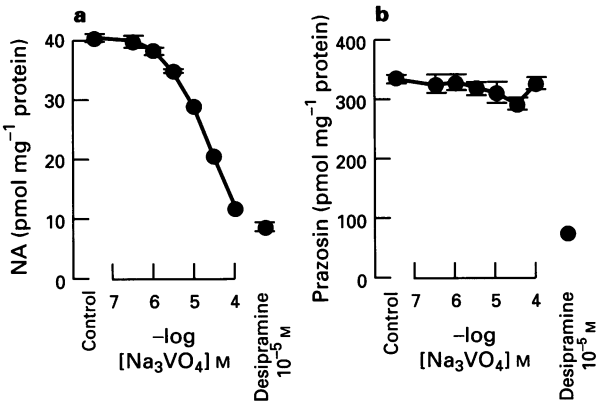

Figure 4 Effects of the P-ATPase inhibitor sodium orthovanadate (Na_3VO_4) on the uptake of amines in SK-N-SH cells (a) and in GnRH cells (b). Vanadate inhibited the uptake of (−)-noradrenaline in the SK-N-SH cells (IC_{50} 3.6×10^{-5} M). This value is similar to the IC_{50} which was reported for the inhibitory effect of sodium orthovanadate on the P-ATPase sodium pump in erythrocytes (IC_{50} 4×10^{-5} M; Cantley *et al.*, 1978). In contrast, vanadate had no effect on the uptake of prazosin in the GnRH cells, confirming that uptake in these cells is independent of sodium. In these experiments, the incorporation of amines was inhibited by desipramine, confirming that active uptake was taking place.

The role of protons in amine uptake The V-ATPase inhibitor, bafilomycin A_1, inhibited the uptake of (−)-noradrenaline into the SK-N-SH cells (data not shown). Bafilomycin A_1 also inhibited the uptake of prazosin in the GnRH cells (Figure 5). At the highest concentration that was used (10^{-6} M), the inhibitory effect of bafilomycin A_1 on the uptake of prazosin in the GnRH cells was similar to the effect of desipramine 10^{-5} M (Figure 5).

The monovalent (H^+/Na^+) carboxylic ionophore, monensin, inhibited the uptake of (−)-noradrenaline in the SK-N-SH cells (IC_{50} 2.5×10^{-7} M; data not shown) and the uptake of prazosin in the GnRH cells (IC_{50} 10^{-6} M; Figure 5). In these experiments, the accumulation of amines was inhibited by desipramine 10^{-5} M, confirming that uptake was taking place (Figure 5).

The organic base, chloroquine, inhibited the uptake of (−)-noradrenaline (at 10^{-6} M) in the SK-N-SH cells (IC_{50} 8×10^{-6} M) and the uptake of prazosin (10^{-6} M) in the GnRH cells (IC_{50} 6.8×10^{-6} M; Figure 6). In contrast, chloroquine had no effect on the association of prazosin (10^{-6} M) with COS-7 cells (Figure 6). In these experiments, desipramine 10^{-5} M inhibited the uptake of (−)-noradrenaline 10^{-6} M in the SK-N-SH cells and the uptake of prazosin 10^{-6} M in the GnRH cells. Desipramine 10^{-5} M had no effect on the accumulation of prazosin in the COS-7 cells (data not shown).

Extracellular pH was varied from 7.40 to 8.10 by increments of 0.10 units. Increases in extracellular pH in the range 7.50 to 8.10 were associated with reduction in the uptake of prazosin in the GnRH cells and of the uptake of (−)-noradrenaline in the SK-N-SH cells (Table 1). At pH 8.1, the uptake of prazosin was 54% and the uptake of (−)-noradrenaline was 68% of the appropriate control value at pH 7.40 (Table 1).

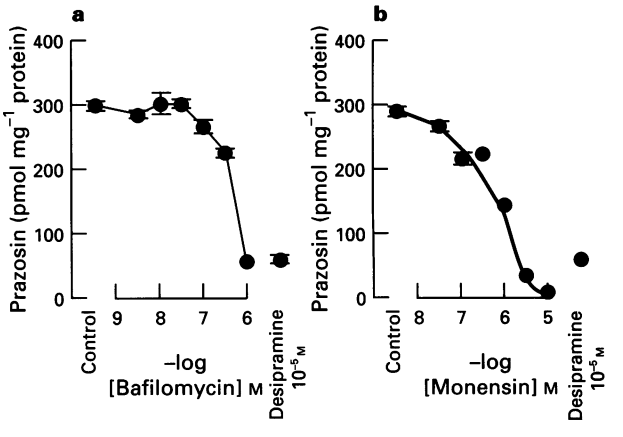

Figure 5 (a) Inhibition of the uptake of prazosin in GnRH cells by the V-ATPase inhibitor, bafilomycin A_1. At the highest concentration that was used (10^{-6} M), the inhibitory effect of bafilomycin A_1 was indistinguishable from that of desipramine. This indicates that uptake in these cells derives its energy from a proton pump. In the concentrations used in the present study, bafilomycin A_1 does not inhibit P-ATPase ion pumps, and F-ATPase pumps, including mitochondrial ATPase, are completely resistant to this drug (Bowman *et al.*, 1988). (b) Inhibition of the uptake of prazosin in GnRH cells by the monovalent (H^+/Na^+) carboxylic ionophore, monensin. The inhibitory effect of monensin, which is exerted within the range of concentrations at which it increases intracellular pH (Maxfield, 1982), indicates that uptake of prazosin in the GnRH cells is into an acidified intracellular compartment. The data are compatible with the conclusion from experiments with bafilomycin A_1.

Effects of reserpine on the uptake of amines in GnRH and SK-N-SH cells

In the SK-N-SH cells, reserpine inhibited the retention of (−)-noradrenaline 10^{-6} M in a concentration-dependent manner (IC_{50} 1.3×10^{-8} M; Figure 7). In contrast, in the GnRH cells studied in an identical manner, reserpine had no consistent effect on the retention of prazosin (Figure 7; prazosin uptake at 60 min in GnRH cells: control: 305.1 ± 10.7; reserpine 10^{-7} M: 313.3 ± 8.5 pmol mg^{-1} protein).

Effects of steroid hormones on the uptake of prazosin in GnRH cells

The uptake of prazosin in the GnRH cells was unaffected by hydrocortisone, corticosterone, β-oestradiol, progesterone or testosterone in concentrations of 10^{-5} M (data not shown). Two separate experiments were carried out, in which the steroid hormones were added in 10 fold excess over prazosin (concentrations of steroids 10^{-5} M; prazosin 10^{-6} M) and in 5000 fold excess over prazosin (steroids 10^{-5} M; prazosin 2×10^{-9} M).

Effect of unlabelled prazosin on the binding of [^{3}H]-prazosin to COS-7 cells transfected with α_1-adrenoceptor cDNA

In transfected COS cells, [^{3}H]-prazosin was displaced by unlabelled prazosin (IC_{50} 2.3×10^{-9} M), but no increase in binding of the radioligand was evident above 10^{-7} M (Figure 7).

Discussion

Neurones accumulate amine neurotransmitters by two active transport processes; carrier molecules in the plasma membranes of presynaptic nerve terminals recapture released neurotransmitters from the extracellular synaptic space into the cytoplasm. A second set of carriers in the membranes of neurosecretory vesicles then transport the recaptured amines from the cytoplasm for storage in the vesicles (Iversen, 1967; Axelrod, 1971). In addition to transport into presynaptic nerve terminals, neurotransmitters may also be accumulated in some non-neuronal cells, such as myocytes and glia (Iversen, 1965; Henn & Hamberger, 1971; Hosli & Hosli, 1978; Kimelberg & Pelton, 1983; Bouvier *et al.*, 1992; Pines *et al.*, 1992; Storck *et*

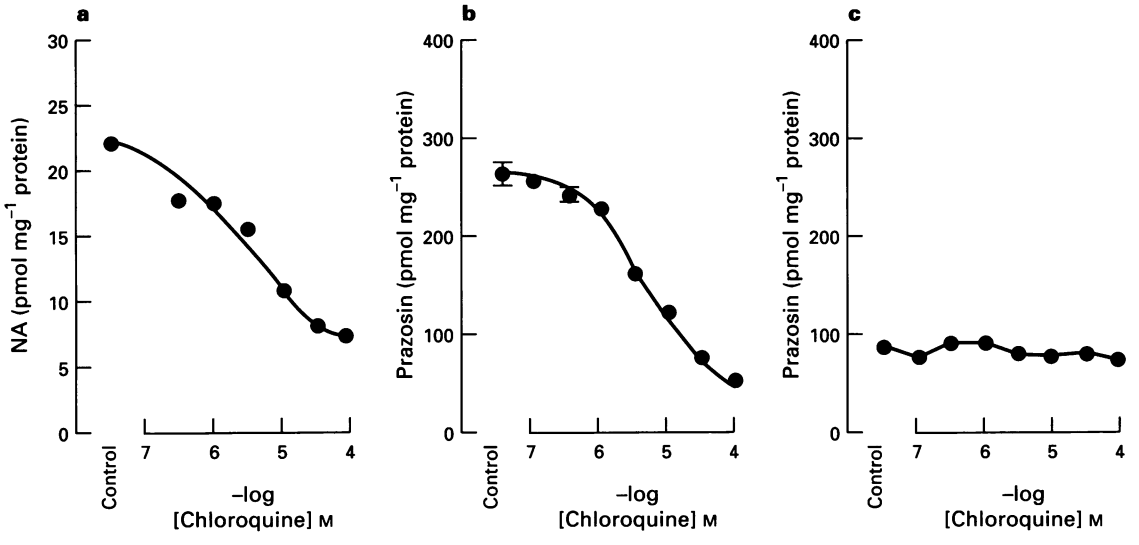

Figure 6 Effects of the organic base chloroquine on the uptake of amines in SK-N-SH cells (a), GnRH cells (b) and COS-7 cells (c). Chloroquine inhibited the uptake of (−)-noradrenaline (10^{-6} M) in the SK-N-SH cells, presumably by increasing the pH of neurosecretory vesicles which store noradrenaline in these cells: IC_{50} 8×10^{-6} M. Chloroquine also inhibited uptake of prazosin (10^{-6} M) in GnRH cells (b) (IC_{50} 6.8×10^{-6} M). This effect of chloroquine which is exerted within the range of concentrations at which it alters intracellular pH (Ohkuma & Poole, 1978; Maxfield, 1982), confirms that uptake of prazosin in these cells is dependent on a proton gradient. The absence of a comparable inhibitory effect in COS cells suggests that accumulation of prazosin in the GnRH cells is unlikely to be into acidified particles which are present in all eukaryotic cells, but is more likely to be into some specialised neuronal compartment, such as neurosecretory vesicles or internalised clathrin-coated pits.

Table 1 Effect of extracellular pH on the uptake of prazosin in GT1-1 GnRH cells and (-)-noradrenaline in SK-N-SH neuroblastoma cells

pH	*Prazosin* (pmol mg^{-1}) *in GT1-1 cells*	*% control*	*Noradrenaline* (pmol mg^{-1}) *in SK-N-SH*	*% control*
7.40	292.8 ± 10.1	100%	33.8 ± 0.2	100%
7.50	306.5 ± 14.9	104%	34.4 ± 0.1	101%
7.60	278.6 ± 12.6	95%	32.5 ± 0.4	96%
7.70	285.0 ± 11.2	97%	32.2 ± 0.1	95%
7.80	249.4 ± 11.3	85%	30.4 ± 0.5	90%
7.90	210.4 ± 5.9	71%	27.2 ± 0.0	80%
8.00	193.4 ± 10.9	66%	23.8 ± 0.2	70%
8.10	159.8 ± 15.4	54%	23.1 ± 0.1	68%

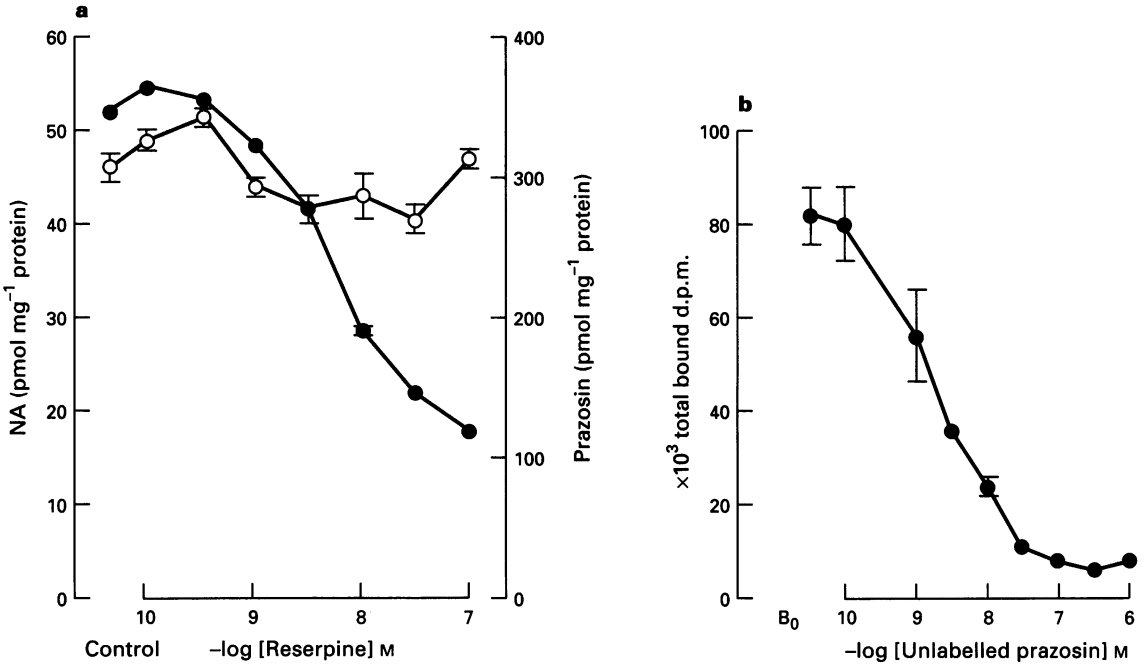

Figure 7 (a) Effects of reserpine, an inhibitor of vesicular amine transporters, on the uptake of amines in SK-N-SH neuroblastoma cells and in GT1-1 GnRH cells. Reserpine inhibited the uptake of (−)-noradrenaline 10^{-6} M in the SK-N-SH cells (●) (IC_{50} 1.3×10^{-8} M) with a potency similar to that in membranes of CHO cells which had been transfected with the chromaffin cell vesicular amine transporter (IC_{50} 2.5×10^{-8} M; Liu *et al.*, 1992). Reserpine had no effect on the uptake of prazosin 10^{-6} M in the GnRH cells (○) which were studied in an identical manner. The insensitivity to reserpine and blockade by desipramine distinguish the uptake of prazosin from the known vesicular amine transporters. (b) Effect of unlabelled prazosin on the binding of [^{3}H]-prazosin to intact COS-7 cells transfected with α_{1B}-adrenoceptor cDNA. The cells were incubated at 37°C for 60 min with [^{3}H]-prazosin 2.3×10^{-9} M, with or without unlabelled prazosin in the indicated concentrations. Unlabelled prazosin displaced [^{3}H]-prazosin from the receptors but there was no increase in the binding of the radioligand. This indicates that the paradoxical increase that was observed in the hypothalamic cells was unlikely to be due solely to some function of α_1-adrenoceptors, such as internalisation of ligand-receptor complexes. The data represent total bound d.p.m.

al., 1992). Thus, uptake processes were known to exist in presynaptic nerve terminals and in some non-neuronal cells; our work provided the first evidence for uptake in postsynaptic neurones (Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al.*, 1993).

This novel amine uptake process in postsynaptic neurones is characterized by a paradoxical increase in accumulation of [^{3}H]-prazosin in the presence of concentrations of unlabelled prazosin greater than 10^{-7} M (Figure 1; Al-Damluji *et al.*, 1993). The following evidence indicates that the increase in accumulation of [^{3}H]-prazosin is due to an energy-requiring, active uptake process: (1) The paradoxical increase was abolished by desipramine (Al-Damluji *et al.*, 1993; Figure 1), which is known to inhibit neuronal amine uptake (Graefe & Bonisch, 1989). (2) The small amount of prazosin accumulated in non-neuronal COS-7 cells was unaffected by desipramine, and there was no paradoxical increase in accumulation of [^{3}H]-prazosin in these COS-7 cells (Figure 1). This indicated that the increase in association of prazosin with GnRH cells was unlikely to be due to non-specific accumulation in cellular membranes or intracellular compartments which are present in all eukaryotic cells. The increase apparently requires the presence of a desipramine-blockable uptake process which is present in GnRH cells but not in COS-7 cells (Figure 1). (3) In membrane preparations of GnRH cells, there was no increase in binding of [^{3}H]-prazosin in the presence of high concentrations of unlabelled prazosin, indicating that the increase requires the presence of intact cells or organelles (Al-Damluji *et al.*, 1993). (4) Cooling the cells inhibited the incorporation of prazosin 2×10^{-9} M into GnRH cells and abolished the paradoxical increase in accumulation of [^{3}H]-prazosin (Figure 2).

SK-N-SH cells possess uptake processes which are normally present in presynaptic neurones. Thus, uptake and storage of noradrenaline were inhibited by desipramine (IC_{50} 1.7×10^{-8} M) and reserpine (IC_{50} 1.3×10^{-8} M; Figure 7) at potencies similar to their potencies in the cloned presynaptic uptake$_1$ noradrenaline transporter and the vesicular amine transporter (Pacholczyk *et al.*, 1991; Liu *et al.*, 1992). In SK-N-SH cells, increasing concentrations of unlabelled noradrenaline did not increase the binding of [^{3}H]-noradrenaline (Figure 2). These comparative observations illustrate the paradoxical nature of the findings on prazosin and indicate that the increase is not a general property of neuronal transporters.

As uptake of prazosin appeared to be an energy-requiring process, we attempted to identify the source of energy. The plasma membrane neurotransmitter transporters derive their energy from the electrochemical gradient of sodium that is generated by the Na^+/K^+-ATPase ('sodium pump'; Pedersen & Carafoli, 1987; Nelson, 1991). Thus, uptake of neurotransmitters such as noradrenaline is absolutely dependent on the presence of sodium in the extracellular space (Figure 3; Iversen & Kravitz, 1966; Pacholczyk *et al.*, 1991). In contrast, in GnRH cells studied in an identical manner, exclusion of sodium caused only a minor reduction of prazosin uptake; in the absence of sodium, desipramine-blockable uptake was evident, representing 70% of the uptake of prazosin at 60 min (Figure 3). Thus, uptake of prazosin in GnRH cells, unlike the known plasma membrane neurotransmitter transporters, is not absolutely dependent on the presence of sodium.

Vanadate inhibits P-ATPase pumps, including the Na^+/K^+-ATPase (Cantley *et al.*, 1978; Pedersen & Carafoli, 1987). Vanadate diminished the uptake of noradrenaline in SK-N-SH cells but had no effect on the uptake of prazosin in GnRH cells (Figure 4). This confirmed that prazosin uptake in GnRH cells is not dependent upon energy from the electrochemical gradient of sodium or a P-ATPase.

Vesicular neurotransmitter transporters derive their energy from the electrochemical gradient of protons generated by V-

ATPase in neurosecretory vesicles (Bashford *et al.*, 1975; Pedersen & Carafoli, 1987; Nelson, 1991). Bafilomycin A_1, a specific inhibitor of V-ATPase proton pumps (Bowman *et al.*, 1988), completely inhibited prazosin uptake in GnRH cells (Figure 5), suggesting that uptake requires energy from an electrochemical proton gradient generated by a V-ATPase ion pump. Thus, enhanced prazosin accumulation at higher concentrations of the drug may be into an acidified intracellular compartment, such as neurosecretory vesicles. Inhibition of the uptake of prazosin by increases in extracellular pH (Table 1) is compatible with this conclusion.

Further evidence for dependence of prazosin uptake on a proton gradient was obtained from experiments that used monensin and chloroquine. Monensin is a monovalent carboxylic ionophore which forms lipid-soluble complexes with cations. It traverses the lipid phase of cellular membranes, resulting in movement of sodium ions into cells, in exchange for protons (Pressman & Fahim, 1982; Ledger & Tanzer, 1984). Chloroquine is an organic base which diffuses rapidly into intracellular acidified particles (Ohkuma & Poole, 1978). Monensin and chloroquine inhibited prazosin uptake in GnRH cells (Figures 5 and 6), confirming that uptake of prazosin in these cells is likely to be into acidified intracellular particles. In contrast, chloroquine had no comparable inhibitory effect on the accumulation of prazosin in COS-7 cells (Figure 6). This confirmed that accumulation of prazosin in GnRH cells is unlikely to be into acidified particles such as lysosomes or mitochondria which are present in all eukaryotic cells, but is more likely to be into some specialised neuronal compartment, such as neurosecretory vesicles or internalised clathrin-coated pits.

Vesicular amine transporters are insensitive to desipramine but are blocked by reserpine (Liu *et al.*, 1992; Erickson *et al.*, 1992). Reserpine inhibited noradrenaline uptake in SK-N-SH cells but had no effect on prazosin uptake in GnRH cells (Figure 7). The resistance to reserpine and blockade by desipramine indicated that prazosin uptake in GnRH cells is not via a known vesicular amine transporter.

Noradrenaline is removed by some non-neuronal cells via $uptake_2$ (Iversen, 1965). This process is insensitive to desipramine and is not sodium-dependent but is blocked by steroid hormones (Iversen & Salt, 1970; Salt, 1972). Uptake of prazosin by GnRH cells was unaffected by high concentrations of steroid hormones. The lack of effects of steroids and the sensitivity to antidepressants distinguish the uptake process in GnRH cells from $uptake_2$.

Lysosomotropic drugs are cationic amphiphilic compounds which exist as weak bases at physiological pH. They enter cells by diffusion in unprotonated form. They become protonated in the acidic environment of lysosomes, where they remain trapped due to their inability to diffuse through the lipid phase of the membrane (De Duve *et al.*, 1974). The pK_a of prazosin is 6.8 (Alabaster *et al.*, 1987), which makes it an unlikely candidate for a lysosomotropic drug. Lysosomes are present in all eukaryotic cells (Alberts *et al.*, 1989) but the organic base, chloroquine, did not reduce the association of prazosin with COS-7 cells (Figure 6). This confirmed that prazosin is unlikely to accumulate as a result of lysosomotropic properties. Further, the lysosomotropic effect is accelerated by increasing extracellular pH, due to an increase in the proportion of the compound that is unprotonated and therefore able to diffuse into cells (De Duve *et al.*, 1974; Marini *et al.*, 1992). However, uptake of prazosin was not enhanced by increasing extracellular pH (Table 1). It therefore seems unlikely that uptake of prazosin in GnRH cells is due to a lysosomotropic effect.

We previously reported that uptake of noradrenaline in GnRH cells was unaffected by steroids and was reduced by 30% by exclusion of sodium (Al-Damluji *et al.*, 1993). It therefore seems possible that the same uptake process transports prazosin and smaller amounts of noradrenaline. GnRH cells accumulate some other compounds acting at α_1-adrenoceptors (Al-Damluji & Kopin, unpublished observations), so we tested the possibility that the increase in the apparent binding of [^{3}H]-prazosin was due to association with α_1-adrenoceptors. However, in COS-7 cells transfected with α_1-adrenoceptor cDNA, [^{3}H]-prazosin was displaced by unlabelled prazosin but no increase of the radioligand was evident above 10^{-7} M (Figure 7). This indicated that the paradoxical increase was unlikely to be due solely to some function of α_1-adrenoceptors, such as internalisation of ligand-receptor complexes.

Thus, GnRH neurones accumulate prazosin in nanomolar concentrations by an energy-dependent, desipramine-blockable process. Uptake is due to a carrier which is activated by increasing concentrations of prazosin, resulting in an increase in accumulation of [^{3}H]-prazosin. We believe that this is the first neuronal uptake process which has been shown to be activated by its substrate. This carrier is distinguishable from known amine transporters. While it resembles plasma membrane transporters in that it is blocked by tricyclic antidepressants, it differs from these transporters by its independence of sodium and reliance on protons for a source of energy. Uptake of prazosin differs from vesicular transporters by its insensitivity to reserpine and blockade by antidepressants. The properties of this process and its novel location in postsynaptic neurones make it an interesting subject for further studies.

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(Received March 22, 1995
Revised August 9, 1995
Accepted September 21, 1995)