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Visual detection of transport-P in peptidergic neurones

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1 Hypothalamic peptidergic neurones possess an uptake process for amines (transport-P), for which prazosin is a substrate. It is characterized by a paradoxical increase in the accumulation of [^{3}H]-prazosin when the concentration of unlabelled prazosin is increased above 10^{-7} M. This increase is due to activation of a proton-dependent, vacuolar-type ATPase-linked pump that is blocked by tricyclic antidepressants. This study utilized a fluorescence method to detect amine uptake in individual cells.

2 Prazosin is fluorescent but most of its emission spectrum is in the ultraviolet range. We therefore used an analogue of prazosin in which the furan ring had been substituted with a fluorescent group, BODIPY FL. This compound's emission maximum is in the green part of the visible spectrum.

3 BODIPY FL prazosin accumulated in immortalised peptidergic neurones and the characteristic emission spectrum of the compound was evident in these cells. Accumulation of BODIPY FL prazosin was saturable and was inhibited by the tricyclic antidepressant desipramine and by unlabelled prazosin. As previously described for prazosin, uptake of BODIPY FL prazosin was blocked by cold temperature and by the organic base chloroquine. Thus, prazosin and BODIPY FL prazosin were accumulated by the same uptake process.

4 BODIPY FL prazosin accumulated in a granular distribution, which is compatible with storage in intracellular vesicles.

5 Hypothalamic cells from foetal rats in primary culture also accumulated BODIPY FL prazosin by a desipramine-sensitive process. Uptake was predominantly in neurones and glial cells did not accumulate the amine.

6 Fluorescent detection provides visual evidence for amine uptake in peptidergic neurones and should enable detailed study of the distribution of this process in the brain.

Keywords: Biological transport; uptake; hypothalamus; prazosin; desipramine

Introduction

The peptidergic neurones of the hypothalamus are densely innervated by noradrenergic nerve terminals and noradrenaline plays a physiological role in regulating the neuroendocrine functions of the hypothalamus (for reviews, see Al-Damluji, 1993; Pacak *et al*., 1995). In rats, noradrenergic neurones regulate the secretion of hypothalamic gonadotrophin-releasing hormone (GnRH) which controls reproductive function (Kalra & Crowley, 1992; Al-Damluji, 1993).

The amine and amino acid neurotransmitters are inactivated predominantly by re-uptake into the presynaptic nerve terminals and into neighbouring glial cells (Iversen, 1967; Axelrod, 1971). Presynaptic nerve terminals possess two sequential uptake processes: transporter molecules that are located in the plasma membrane accumulate neurotransmitter molecules from the extracellular synaptic space into the cytoplasm by a process that requires an electrochemical gradient of sodium ions and is blocked by tricyclic antidepressants (Amara & Kuhar, 1993; Lester *et al*., 1994). A second transporter system is located in the membranes of neurosecretory vesicles. These vesicular transporters utilize the electrochemical gradient of protons that is generated by a vacuolar-type ATPase (V-ATPase), to sequester neurotransmitters from the cytoplasm into the vesicles. Vesicular amine transporters are blocked by reserpine but are insensitive to antidepressants (Edwards, 1992; Erickson *et al*., 1992). The uptake processes in non-neuronal cells are independent of ion gradients and insensitive to antidepressants and reserpine, but are blocked by steroid hormones (Iversen & Salt, 1970; Salt, 1972).

In addition to the uptake processes in presynaptic nerve terminals and in non-neuronal cells, we have described an uptake process for amines in postsynaptic (peptidergic) neurones (Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al*., 1993). This uptake process became evident while we were examining hypothalamic neurones for the presence of α_1-adrenoceptors with the α_1-adrenoceptor ligand [^{3}H]-prazosin. In primary hypothalamic cell cultures and in a cell line of GnRH neurones, [^{3}H]-prazosin bound to the cells and was displaced by unlabelled prazosin in the concentration range 10^{-9} to 10^{-7} M. However, when the concentration of unlabelled prazosin was increased above 10^{-7} M, there was a paradoxical increase in accumulation of [^{3}H]-prazosin which could be abolished by desipramine. In the presence of desipramine, only displacement of [^{3}H]-prazosin by unlabelled prazosin was seen. The paradoxical increase in accumulation of [^{3}H]-prazosin was not seen when hypothalamic cells were studied in the cold, or in membrane preparations of hypothalamic neurones (Al-Damluji *et al*., 1993; Al-Damluji & Kopin, 1996a). These findings were interpreted as indicating the presence of α_1-adrenoceptors and an uptake process for prazosin. As the concentration of unlabelled prazosin is increased, [^{3}H]-prazosin is displaced from the receptors. The uptake process becomes evident at higher concentrations of unlabelled prazosin, because this uptake process is activated by its substrate (prazosin). Desipramine-inhibitable uptake is detectable at nanomolar concentrations of prazosin, but as the concentration of unlabelled prazosin is increased, there is further activation of uptake. This activation results in the increase in accumulation of [^{3}H]-prazosin, despite the reduction of specific activity of [^{3}H]-prazosin, consequent upon dilution with unlabelled prazosin (Al-Damluji & Kopin, 1996a). Uptake of prazosin and the paradoxical increase are active, energy-

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dependent processes, requiring an electrochemical gradient of protons that is generated by a V-ATPase (Al-Damluji & Kopin, 1996a).

The paradoxical increase in accumulation of the radioligand in peptidergic neurones was not seen when presynaptic neurones were studied in an identical manner (Al-Damluji & Kopin, 1996a). This indicated that the increase was not a general property of neurotransmitter transporters. Uptake of prazosin in peptidergic neurones (transport-P) was distinguishable from presynaptic plasma membrane transporters by its independence of sodium and its reliance on protons for a source of energy (Al-Damluji & Kopin, 1996a). Although transport-P resembles the presynaptic vesicular amine transporters by its dependence on protons and V-ATPase, it differs from these transporters by its insensitivity to reserpine and blockade by antidepressants (Al-Damluji & Kopin, 1996a,b). The lack of effects of steroid hormones and sensitivity to antidepressants also differentiated transport-P from the uptake processes in non-neuronal cells (Al-Damluji & Kopin, 1996a,b). Thus, transport-P was distinguishable from other uptake processes by its anatomical location and by its functional properties.

In this study, we have utilized fluorescence methods to examine the hypothesis that peptidergic neurones accumulate amines by a desipramine-sensitive process. The findings provide visual evidence for the presence of transport-P in peptidergic neurones.

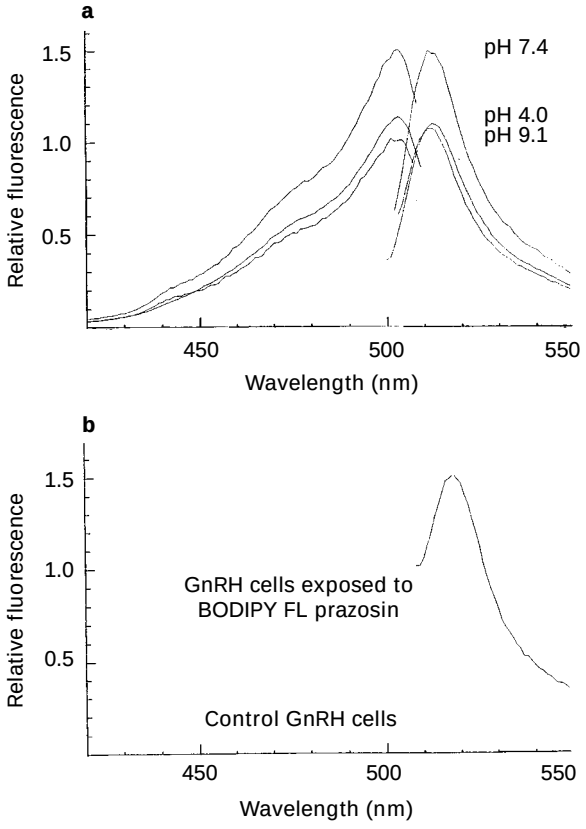

Figure 1 (a) Excitation (left) and emission spectra (right) of BODIPY FL prazosin in solution, at different pH values. The wavelength of the emission maximum (512 nm) is in the green part of the visible spectrum and is unaffected by changes in pH in the range 4.0 to 9.1. Fluorescence intensity is greatest at pH 7.4. (b) GnRH cells were grown on a glass coverslip coated with poly-D-lysine and laminin. Autofluorescence is minimal before exposure to BODIPY FL prazosin (control GnRH cells). The cells were then incubated in the presence of BODIPY FL prazosin 1.77×10^{-7} M for 60 min at 37°C, washed, placed in a cuvette and excited with a wavelength of 450 nm. The emission spectrum characteristic of BODIPY FL prazosin is seen in the cells, indicating that they had accumulated the compound.

Methods

Cell culture

Immortalized GT1-1 GnRH neuronal cells were cultured as previously described in detail (Al-Damluji *et al*., 1993). Briefly, the cells were grown in Corning 75 cm^2 flasks in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (ratio 1 : 1) containing 10% FBS, sodium bicarbonate 3.7 g l^{-1} and gentamicin 100 mg l^{-1}, in a humidified atmosphere containing 5% CO_2 in air. Culture media were changed at 48 h intervals. When the cells reached confluence, they were dispersed in the presence of trypsin and DNAseI and grown on glass cover slips or glass chamber slides coated with poly-D-lysine (5 μg cm^{-2}; Sigma P-6407; MW 70,000–150,000) and laminin (0.5 μg cm^{-2}; Sigma L-2020). In some experiments, serum-free medium was used, consisting of DMEM and F12 (ratio 1 : 1), insulin 5 mg l^{-1}, progesterone 6.2 μg l^{-1}, selenium 5.1 μg l^{-1}, putrescine 16.11 mg l^{-1}, arachidonic acid 1 mg l^{-1}, docosahexaenoic acid 0.5 mg l^{-1}, bovine transferrin 100 mg l^{-1}, sodium bicarbonate 3.7 g l^{-1} and gentamicin 100 mg l^{-1}.

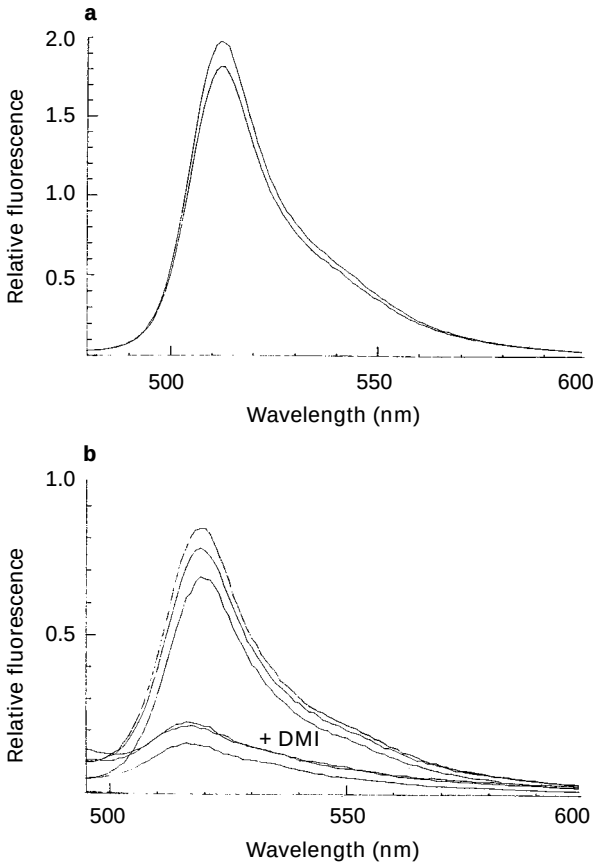

Figure 2 Effect of desipramine on the uptake of BODIPY FL prazosin in GnRH cells. (a) Emission spectrum of BODIPY FL prazosin in solution, with and without desipramine 10^{-5} M. Desipramine had a negligible effect on the emission spectrum; the wavelength of the emission maximum was unchanged but there was a slight increase in fluorescence intensity in the presence of desipramine. Excitation wavelength was 450 nm. (b) GnRH cells grown on glass coverslips were incubated for 60 min at 37°C in the presence of BODIPY FL prazosin 1.77×10^{-7} M, with or without desipramine 10^{-5} M (DMI). The cells were then washed, placed in a cuvette and excited with a wavelength of 450 nm. Bodipy FL prazosin accumulates in the GnRH cells, as indicated by the characteristic emission spectrum,. Accumulation of the compound is blocked by desipramine, indicating that uptake of the fluorescent analogue has similar properties to uptake of prazosin in GnRH cells. Three replicates were performed for each experiment.

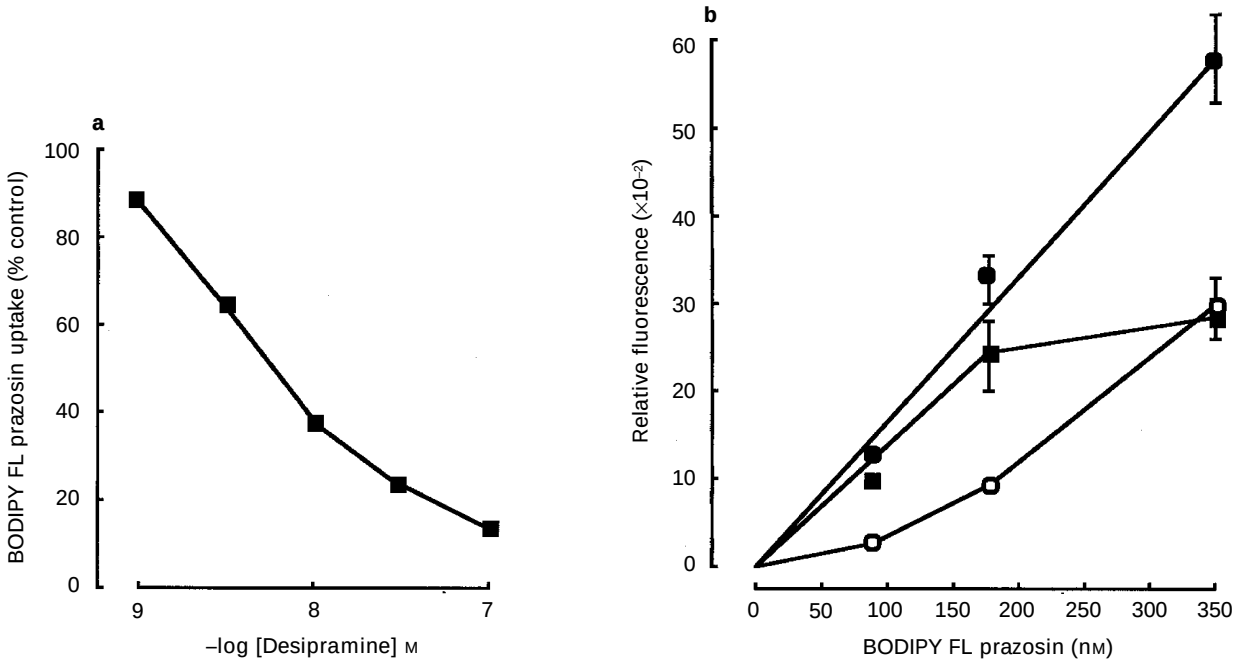

Figure 3 (a) Effects of increasing concentrations of desipramine on the uptake of BODIPY FL prazosin 1.77×10^{-7} M in GnRH cells. Each point represents the mean of three replicates. Standard error bars were smaller than the size of the symbols. $IC_{50} = 8 \times 10^{-9}$ M. (b) Uptake of increasing concentrations of BODIPY FL prazosin in GnRH cells. Specific uptake (■) was defined as total uptake (●) − uptake in the presence of desipramine 10^{-5} M (○). Specific uptake begins to saturate in concentrations greater than 1.77×10^{-7} M. Data are means of three replicates; the vertical lines show s.e.mean. The curves were fitted manually.

Hypothalamic cell cultures from day 18 foetal rats were prepared as previously described in detail (Al-Damluji *et al*., 1993). Briefly, the hypothalami were dissected and the cells were dispersed mechanically in the presence of collagenase and DNAseI. The cells were then washed, dispersed in culture medium containing 10% FBS (250,000 cells ml^{-1}) and incubated in glass chamber slides coated with poly-D-lysine and laminin at a density of 125,000 cells cm^{-2}.

Spectrophotofluorimetry

Compounds were dissolved in Krebs-Ringer-HEPES buffer (KRH buffer, composition in mM: NaCl 125, KCl 4.8, $MgCl_2.6H_2O$ 0.5, Na_2HPO_4 0.7, NaH_2PO_4 1.5, $CaCl_2.2H_2O$ 2.5, glucose 10 and HEPES 23; pH 7.4). Fluorescence was examined in acrylic cuvettes in an SLM Amino 8000 spectrophotofluorimeter. Excitation and emission polarisers were both set horizontal to suppress scattered light and slits were 8 nm wide. The excitation spectra of prazosin and BODIPY FL prazosin were examined with emission wavelengths of 400 nm and 520 nm, respectively, and the emission spectra of these two compounds were examined with excitation wavelengths of 340 nm and 490 nm, respectively. Fluorescence of these compounds was measured relative to a rhodamine standard.

Fluorescent detection of amine uptake in GT1-1 GnRH cells

GT1-1 GnRH cells were dispersed in culture medium at a density of 10^6 cells ml^{-1} and were grown at a density of 4×10^5 cells cm^{-2} on glass coverslips coated with poly-D-lysine and laminin. Studies were carried out after two days in culture, when the cells had become confluent. The cell-covered coverslips were washed in KRH buffer, then placed in a cuvette containing 3 ml KRH buffer, at an angle of 45° to the incident light, such that specular reflection would be diverted away from the detector. Autofluorescence was then measured, with an excitation wavelength of 450 nm. This non-peak wavelength was chosen to reduce further interference from light scattered on the cell-covered coverslips. Excitation and emission polarizers were horizontal and slits were 8 nm wide. The coverslips were then placed in a cuvette containing 3 ml of BODIPY FL prazosin, with or without the relevant unlabelled compounds and incubated at 37°C for 60 min. The coverslips were then rinsed in 3 ml KRH buffer, placed in another cuvette containing 3 ml fresh KRH buffer and fluorescence was measured as described above.

Fluorescence microscopy

GT1-1 GnRH cells were grown in serum-free medium or in medium containing 10% FBS at a density of 100,000 cells ml^{-1} (25,000 cells cm^{-2} in glass chamber slides coated with poly-D-lysine and laminin. Hypothalamic cells from foetal rats were dispersed in culture medium containing 10% FBS (250,000 cells ml^{-1}) and incubated at a density of 125,000 cells cm^{-2} in glass chamber slides coated with poly-D-lysine and laminin. After 2–3 days in culture, the cells were washed twice with 2 ml of KRH buffer at room temperature, then incubated at 37°C for 60 min in the presence of BODIPY FL prazosin 1.77×10^{-7} M, with or without unlabelled compounds in the indicated concentrations in KRH buffer. The cell-covered chamber slides were then placed on ice and the incubation medium was aspirated. The chambers and gaskets were removed and the slides were washed in ice cold KRH buffer. The cells were then fixed in ice-cold 3.7% formalin (pH 7.0) for 15 min. The cell-covered slides were then dried in a stream of warm air and mounting medium (light, white, mineral oil; Sigma M-3516) was added followed by a glass cover slip. Fluorescence was examined with a Nikon Microphot-FX fluorescence microscope with a 470–490 nm excitation filter, 520–560 nm emission filter and a 510 nm dichroic mirror.

Materials

Immortalized GnRH neuronal cells (GT1-1 cells; Mellon *et al*., 1990) were generously provided by Dr R.I. Weiner (University of California School of Medicine, San Francisco, California). Pregnant (15 days) Sprague-Dawley rats were from Taconic Farms, New York. Heat-inactivated foetal bovine serum (FBS) was obtained from Life Technologies

(Gaithersburg, Maryland) and BODIPY FL prazosin from Molecular Probes (Eugene, California; catalogue number B-7433; lot no. 3241-1). This compound was dissolved in dimethylsulphoxide and stored in aliquots at −20°C in the dark. Two-well glass chamber slides were from Nunc (Roskilde, Denmark; catalogue number 177380; surface area 4 cm^2/well). Acrylic cuvettes were from Sarstedt (Numbrecht, Germany; catalogue number 67.755; $10 \times 10 \times 48$ mm). Unlabelled compounds and culture media were from Sigma (St. Louis, Missouri).

Results

Spectrophotofluorimetry

Prazosin has intrinsic fluorescence but its emission spectrum is predominantly ultraviolet (excitation and emission maxima 340 and 400 nm, respectively; data not shown). In contrast, the emission spectrum of BODIPY FL prazosin was within the visible range (excitation and emission maxima 500 and 512 nm, respectively; Figure 1). Changes in pH in the range 4.0 to 9.1 influenced the intensity of fluorescence but they did not affect the wavelength of the emission maximum of BODIPY FL prazosin (Figure 1). Intensity of fluorescence was greatest at pH 7.4.

With an excitation wavelength of 450 nm and an emission window of 480–600 nm, autofluorescence was minimal in GnRH cells grown on glass coverslips (Figure 1). After incubation in the presence of BODIPY FL prazosin (1.77×10^{-7} M) for 60 min and washing, fluorescence of BODIPY FL prazosin was clearly evident, indicating that the cells had accumulated the compound (Figure 1). The emission maximum of BODIPY FL prazosin in GnRH cells was at 517–518 nm (Figure 1). When the cell-coated coverslips were removed from the cuvette, no fluorescence was detected in the KRH buffer, confirming that the measured fluorescence was in the cell-coated coverslips (not shown).

Desipramine 10^{-5} M had a negligible effect on the emission spectrum of BODIPY FL prazosin in solution; the wavelength of the emission peak was unaffected but the amplitude was slightly increased (Figure 2). Desipramine 10^{-5} M inhibited the accumulation of BODIPY FL prazosin (1.77×10^{-7} M) in GnRH cells (Figure 2). In the presence of desipramine 10^{-5} M, the emission maximum of BODIPY FL prazosin in GnRH cells was at 516–517 nm. The inhibitory effect of desipramine was dose-dependent (IC_{50} 8×10^{-9} M; Figure 3). In a limited experiment, specific uptake of BODIPY FL prazosin in GnRH cells (defined as total uptake − uptake in the presence of desipramine 10^{-5} M) began to saturate at concentrations greater than 1.77×10^{-7} M (Figure 3).

Unlabelled prazosin 10^{-5} M had a negligible effect on the emission spectrum of BODIPY FL prazosin in solution; the wavelength of the emission peak was unaffected but the amplitude was slightly increased (Figure 4). Unlabelled prazosin 10^{-6} M reduced the accumulation of BODIPY FL prazosin 1.77×10^{-7} M in GnRH cells (Figure 4). Desipramine 10^{-7} M almost completely blocked the accumulation of BODIPY FL prazosin, in the presence and absence of unlabelled prazosin (Table 1). In the presence of unlabelled prazosin 10^{-6} M, the emission maximum of BODIPY FL prazosin in GnRH cells was shifted to 522 nm (Figure 4 and Table 1). At concentrations up to 10^{-5} M, unlabelled prazosin did not cause an increase in the accumulation of BODIPY FL prazosin 1.77×10^{-7} M (data not shown).

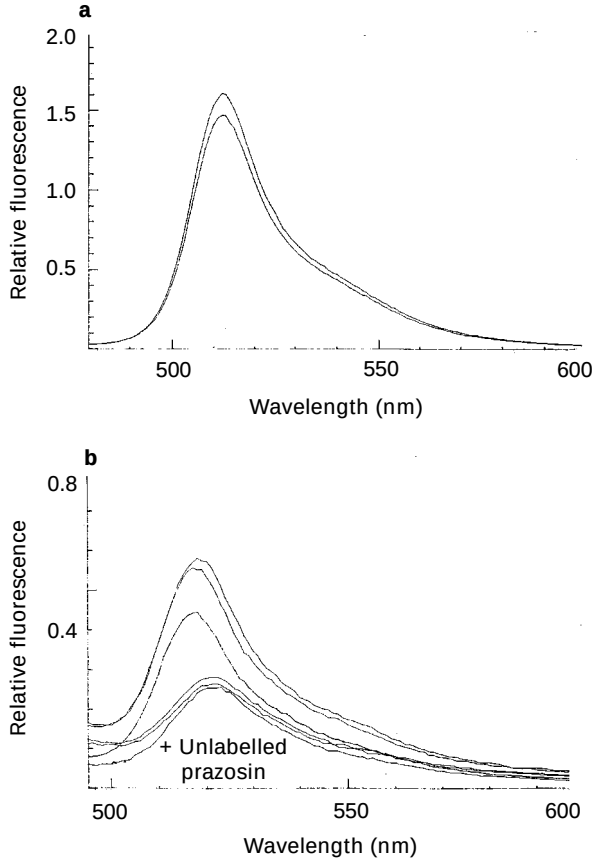

Figure 4 Effect of unlabelled prazosin on the uptake of BODIPY FL prazosin in GnRH cells. (a) Emission spectrum of BODIPY FL prazosin in solution, with and without unlabelled prazosin 10^{-5} M. Unlabelled prazosin had a negligible effect on the emission spectrum; the wavelength of the emission maximum was unchanged but there was a slight increase in fluorescence intensity in the presence of unlabelled prazosin. Excitation wavelength was 450 nm. (b) GnRH cells grown on glass coverslips were incubated for 60 min at 37°C in the presence of BODIPY FL prazosin 1.77×10^{-7} M, with or without unlabelled prazosin 10^{-6} M. The cells were then washed, placed in a cuvette and excited with a wavelength of 450 nm. Bodipy FL prazosin accumulated in the GnRH cells, as indicated by the characteristic emission spectrum. Accumulation of the compound was partially blocked by unlabelled prazosin. Desipramine 10^{-7} M almost completely blocked the accumulation of BODIPY FL prazosin, in the presence or absence of unlabelled prazosin (see Table 1). The results indicate that prazosin and BODIPY FL prazosin are accumulated by the same uptake process in GnRH cells. Three replicates were performed for each experiment.

Table 1 Effects of despiramine (DMI) and unlabelled prazosin (praz) on the accumulation and the wavelength of the emission maximum of BODIPY FL prazosin in GnRH cells

Compound	*Relative fluorescence*	*Emission maximum*
BODIPY FL prazosin 1.77×10^{-7} M in solution		512 nm
In GnRH cells:		
BODIPY FL prazosin 1.77×10^{-7} M	0.34 ± 0.01	518 nm
+ DMI 10^{-7} M	0.06 ± 0.01	517 nm
+ Praz 10^{-6} M	0.11 ± 0.01	522 nm
+ DMI + Praz	0.05 ± 0.02	518 nm

Excitation wavelength was 450 nm. Fluorescence of BODIPY FL prazosin was measured at the emission maximum, relative to rhodamine as standard. Desipramine and unlabelled prazosin had negligible effects on the fluorscence of BODIPY FL prazosin in solution (see Figures 2 and 4).

Fluorescence microscopy

Autofluorescence was minimal in GnRH cells and in hypothalamic cells from foetal rats, with a 470–490 nm excitation filter, 520–560 nm emission filter and a 510 nm dichroic mirror (Figure 5). GnRH cells incubated at 37°C in the presence of BODIPY FL prazosin (1.77×10^{-7} M) acquired an intense green fluorescence, indicating that they had accumu-

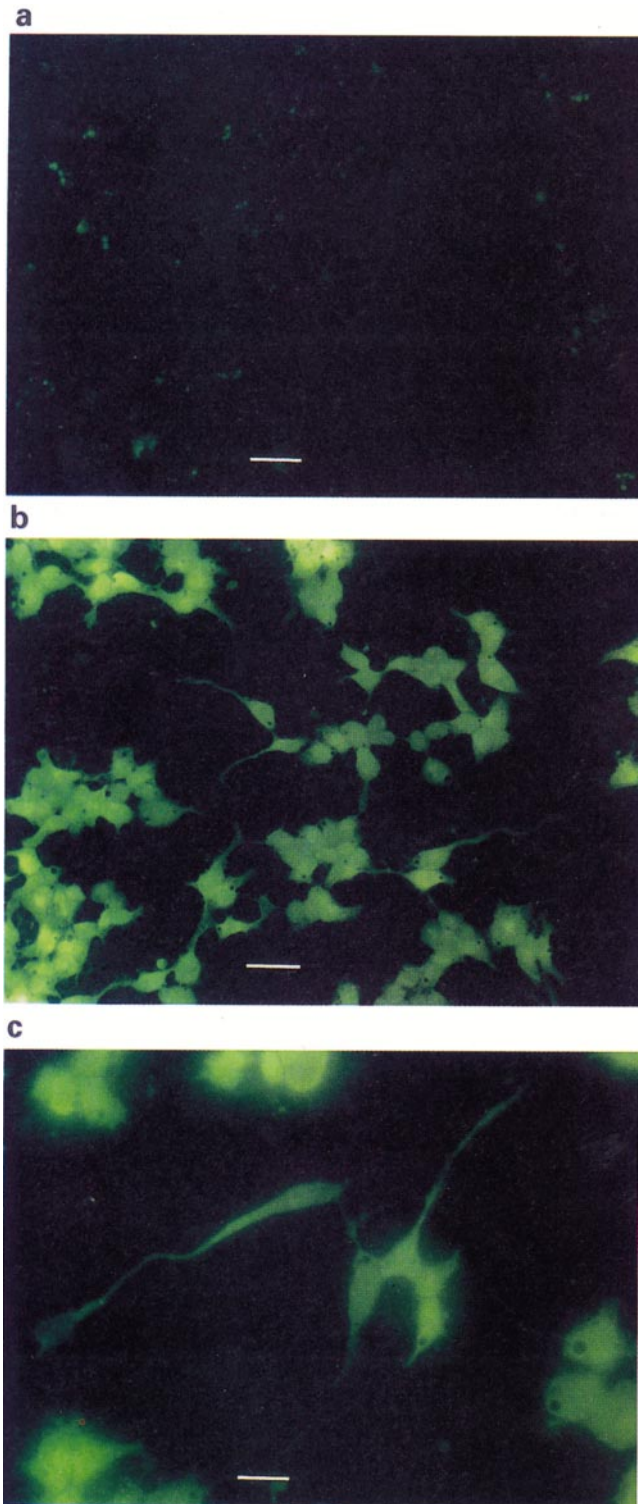

Figure 5 Accumulation of BODIPY FL prazosin in GnRH cells, detected by fluorescence microscopy. GT1-1 GnRH cells were grown in serum-free medium on glass microscope slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of KRH buffer (control, a) or the fluorescent prazosin analogue BODIPY FL prazosin 1.77×10^{-7} M (b and c). The cells were then washed and fixed. Fluorescence microscopy demonstrates that autofluorescence was minimal under these conditions (a). The cells accumulated BODIPY FL prazosin, as indicated by the intense green appearance that is characteristic of this compound (b and c). The scale bars represent 14 μm in (a and b) and 7 μm in (c).

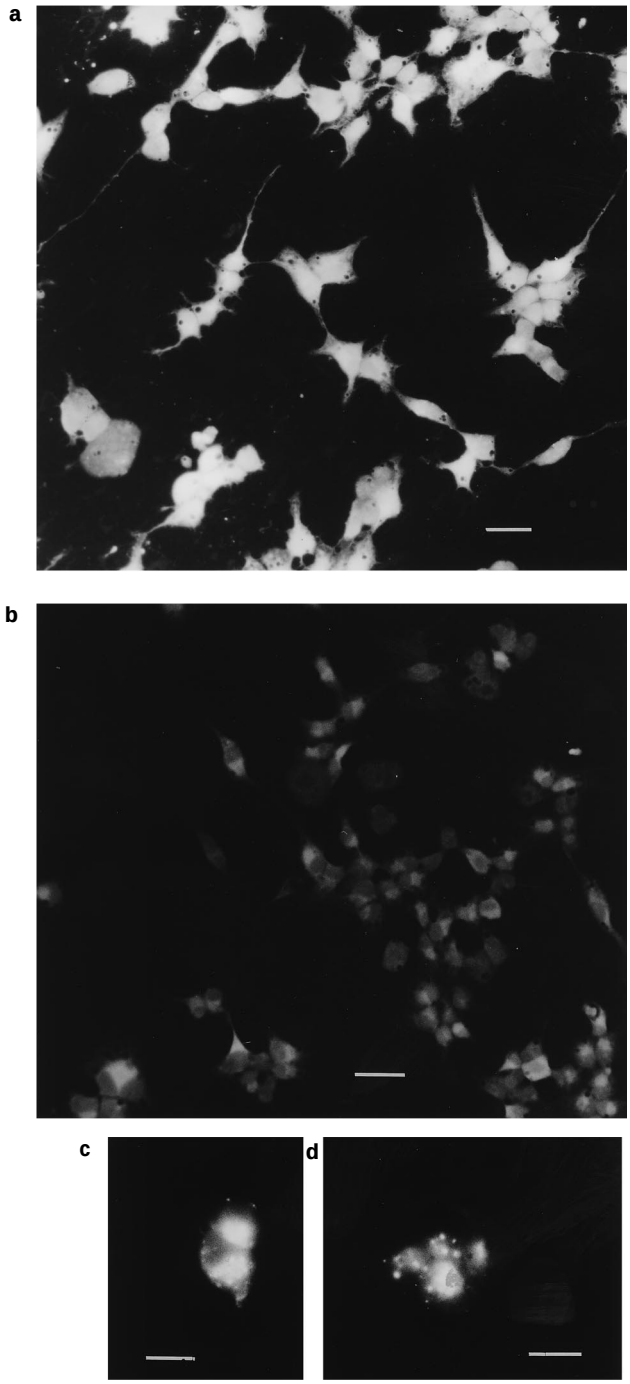

Figure 6 Effects of desipramine and unlabelled prazosin on the accumulation of BODIPY FL prazosin in GnRH cells. GT1-1 GnRH cells were grown in serum-free medium on glass microscope slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of the fluorescent prazosin analogue BODIPY FL prazosin 1.77×10^{-7} M. The cells accumulate the amine (control, a) by a desipramine-inhibitable mechanism (b; desipramine concentration 10^{-5} M). In the lower panels (c and d), the cells were incubated with both BODIPY FL prazosin 1.77×10^{-7} M and unlabelled prazosin 10^{-6} M. This manoeuvre was intended to block binding of BODIPY FL prazosin to α_1-adrenoceptors on the surface of the cells. This reveals that accumulation of BODIPY FL prazosin is in a granular distribution which is compatible with localization in intracellular vesicles. The scale bars represent 14 μm in (a) and (b) and 7 μm in (c) and (d).

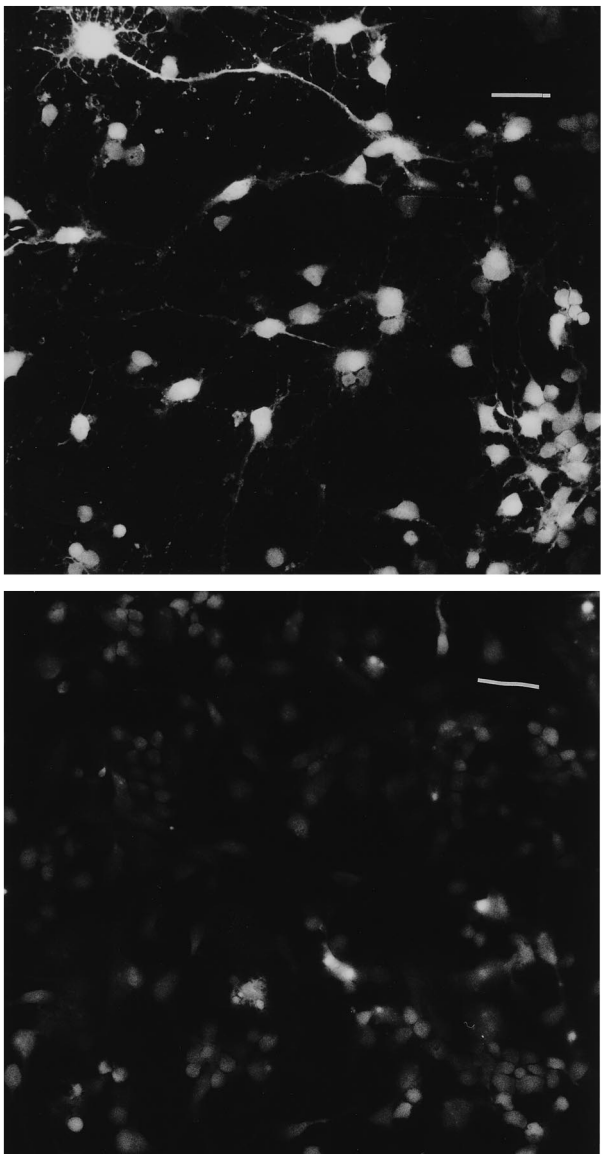

Figure 7 Visual evidence for amine uptake in hypothalamic neurones. Hypothalamic cells from foetal day 18 rats were grown in medium containing 10% FBS on glass microscope slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of the fluorescent prazosin analogue BODIPY FL prazosin 1.77×10^{-7} M, with or without desipramine 10^{-5} M. The cells accumulated the amine (control, a) by a desipramine-inhibitable mechanism (b). Inspection of the intensely labelled cells in (a) reveals that most of them possess neuronal processes with varicosities which are typical of axons and dendrites. This indicates that in the hypothalamus, transport-P is located predominantly in neurones rather than glial cells. The scale bars represent 14 μm in (a) and (b).

lated the compound (Figure 5). The acquisition of intense fluorescence was abolished by desipramine 10^{-5} M (Figure 6). In the presence of desipramine, only a faint fluorescence was evident in these cells (Figure 6). This presumably represents a combination of non-specific binding and binding of BODIPY FL prazosin to α_1-adrenoceptors, as unlabelled prazosin further reduced the faint fluorescence.

In the presence of unlabelled prazosin 10^{-6} M, BODIPY FL prazosin accumulated in GnRH cells in a granular pattern (Figure 6). Accumulation of BODIPY FL prazosin in GnRH cells was inhibited by chloroquine 10^{-4} M and by reducing the incubation temperature from 37°C to 0°C; under these conditions, the appearance of the cells resembled their appearance in the presence of desipramine (Figure 6).

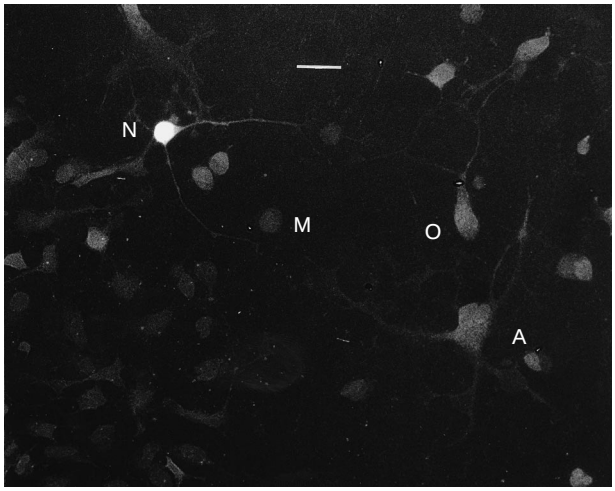

Figure 8 Visual evidence for amine uptake in hypothalamic neurones. Hypothalamic cells from foetal day 18 rats were grown as described in Figure 7, and incubated for one hour in the presence of BODIPY FL prazosin 1.77×10^{-7} M. There is an intensely labelled neurone (N) in the upper left corner. On the right, there is an oligodendrocyte (O) and an astrocyte (A) which are not intensely labelled; their appearance is similar to cells which are incubated in the presence of desipramine (see Figure 7). There are also many round cells (microglia; M) which are not intensely labelled. The findings indicate that in the hypothalamus, transport-P is located predominantly in neurones rather than glial cells. The scale bar represents 14 μm.

Hypothalamic cells from foetal rats also accumulated BODIPY FL prazosin and most of the intensely labelled cells appeared to be neurones (Figures 7 and 8). Accumulation of BODIPY FL prazosin in foetal hypothalamic cells was inhibited by desipramine 10^{-5} M (Figure 7). Glial cells were identifiable in these cultures but they were not intensely labelled with the fluorescent compound. Their faint fluorescence resembled the appearance of neuronal cells incubated in the presence of desipramine (Figures 7 and 8).

Discussion

In previous studies with [^{3}H]-prazosin, we provided evidence for the existence of a new uptake process for amines in postsynaptic (peptidergic) neurones. The present study, in which a fluorescent analogue of prazosin was utilized, extends that work by providing visual evidence for amine uptake in peptidergic neurones.

Prazosin is fluorescent but most of its emission peak was invisible to the human eye. In our studies on the structural requirements for uptake, we found that the furan ring of prazosin was not essential for uptake, but it was required for the paradoxical increase in accumulation of [^{3}H]-prazosin (unpublished observations). Thus, analogues of prazosin that lacked the furan group accumulated in GnRH cells by a desipramine-sensitive process, but they did not display the paradoxical increase in the accumulation of [^{3}H]-prazosin. In this study, we utilized an analogue of prazosin in which the furan ring had been substituted with a fluorescent group (BODIPY FL) whose emission peak is in the green part of the visible spectrum (Figure 1).

Under our experimental conditions, autofluorescence was minimal in GnRH cells examined with both the spectrophotofluorimeter and the fluorescence microscope (Figures 1 and 5). The spectrophotofluorimetric studies demonstrated that these cells accumulated BODIPY FL prazosin by a saturable, desipramine-sensitive process (Figures 2 and 3). Unlabelled prazosin 10^{-6} M partially inhibited the accumulation of BODIPY FL prazosin (Figure 4). As previously described for

prazosin (Al-Damluji & Kopin, 1996a), accumulation of BODIPY FL prazosin in GnRH cells was inhibited in the cold and by the organic base chloroquine. These findings indicated that prazosin and its analogue are internalised by the same desipramine-sensitive, proton-dependent uptake process (transport-P). The absence of a paradoxical increase in accumulation of BODIPY FL prazosin in the presence of unlabelled prazosin (up to 10^{-5} M) is compatible with the previous finding that this increase requires the presence of a furan ring, which is lacking in BODIPY FL prazosin.

Fluorescence microscopy confirmed that GnRH cells accumulate BODIPY FL prazosin by a desipramine-sensitive process; in the presence of desipramine, the intense labelling of GnRH cells was abolished, and only faint labelling was seen (Figure 6). Unlabelled prazosin further reduced the faint labelling. This faint labelling presumably represented a combination of binding of BODIPY FL prazosin to α_1-adrenoceptors, and non-specific binding of this lipophilic compound. Hypothalamic cells from foetal rats also accumulated BODIPY FL prazosin by a desipramine-sensitive process (Figure 7). Most of the intensely-labelled cells apppeared to be neurones, whereas glial cells were not intensely labelled (Figures 7 and 8). These findings indicate that in the hypothalamus, transport-P is located predominantly in neurones, rather than in glial cells.

In previous studies, we had found that accumulation of prazosin in GnRH cells was dependent on an electrochemical gradient of protons and is linked to a V-ATPase (Al-Damluji & Kopin, 1996a). It therefore seemed likely that prazosin was internalized in some acidified intracellular particles, such as neurosecretory vesicles or internalised clathrin-coated pits. In the present study, unlabelled prazosin 10^{-6} M partially inhibited the accumulation of BODIPY FL prazosin (Figure 4). This manoeuvre, which was intended to displace BODIPY FL prazosin from surface α_1-adrenoceptors, revealed that the intracellular distribution of BODIPY FL prazosin was in a granular pattern, presumably representing accumulation in intracellular vesicles (Figure 6).

Although the emission spectrum of BODIPY FL prazosin was similar in solution and in GnRH cells, there was a slight red shift in the emission maximum in GnRH cells. This shift is unlikely to be due to the pH of the intracellular compartment in which BODIPY FL prazosin is stored, as the wavelength of the emission maximum of this compound was insensitive to pH changes in the range 4.0–9.1 (Figure 1). The shift was accentuated by unlabelled prazosin and persisted in the presence of desipramine, indicating that it is unlikely to be due to association of the fluorescent compound with α_1-adrenoceptors or transport-P. The nature of this slight shift is unclear, but may be due to non-specific binding of this compound to various cellular components. The fluorescence microscopy method was insensitive to this slight change in the wavelength of the emission maximum, since the emission filter included wavelengths up to 560 nm.

In conclusion, the present results provide visual evidence for the existence of transport-P in peptidergic neurones. This approach should be useful for detailed studies of the anatomical distribution of this novel uptake process in the brain.

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