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Release of amines from acidified stores following accumulation by Transport-P

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1 Transport-P is an uptake process for amines in peptidergic neurones of the hypothalamus. It differs from other uptake processes by its anatomical location in post-synaptic neurones, its functional properties and by the structure of its ligands. Transport-P accumulates amines in intracellular vesicles, derives its energy from the electrochemical proton gradient and is linked to vacuolar-type ATPase (V-ATPase). Transport-P is blocked by antidepressants. We have now studied the release of amines following uptake by Transport-P in a cell line of hypothalamic peptidergic neurones.

2 Release of prazosin was not inhibited by the antidepressant desipramine; as Transport-P is blocked by desipramine, this indicated that amines are released by a mechanism which is independent of Transport-P.

3 Release of prazosin was sensitive to temperature and conformed to the Arrhenius equation. Release was minimal in the range 0–25°C but accelerated exponentially at higher temperatures up to 33°C. The activation energy for the release of prazosin is 83.1 kJ mol^{-1}, corresponding to a temperature quotient (Q_{10}) value of 3.

4 Release was accelerated by the organic base chloroquine, the ionophore monensin, bafilomycinA1 which inhibits V-ATPase and by increasing extracellular pH. Thus, retention of prazosin requires an intracellular proton gradient which is generated by V-ATPase.

5 Fluorescence microscopy demonstrated that release of BODIPY FL prazosin was temperature dependent and was accelerated by chloroquine and monensin.

6 Thus, following uptake by Transport-P, amines are accumulated in acidified intracellular stores. Their retention in peptidergic neurones requires intracellular acidity. The amines are released by a temperature-dependent process which is resistant to antidepressants.

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Abbreviations: ANOVA, analysis of variance (single factor); DMEM, Dulbecco's modified Eagle's medium; DNAseI, deoxyribonuclease I; Ea, activation energy; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; GnRH, gonadotrophin-releasing hormone; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); IC_{50}, half-maximal inhibitory concentration; KRH, Krebs-Ringer-HEPES buffer; MPP^+, 1-methyl-4-phenylpyridinium; P-ATPase, phosphorylated-type ATPase; Q_{10}, temperature quotient; V-ATPase, vacuolar-type ATPase

Introduction

Transport-P is an unusual uptake process for amines in peptidergic neurones of the hypothalamus. This uptake process became evident while we were examining hypothalamic neurones for the presence of alpha-1 adrenoceptors. The alpha-1 adrenergic ligand [^{3}H]-prazosin binds to peptidergic neurones and is displaced by unlabelled prazosin in concentrations up to 10^{-7} M. However, at concentrations of unlabelled prazosin greater than 10^{-7} M, there is a paradoxical increase in the binding of [^{3}H]-prazosin which can be abolished by antidepressants; in the presence of antidepressants, only displacement of [^{3}H]-prazosin by unlabelled prazosin is seen (Figure 2A; Al-Damluji & Krsmanovic, 1992; Al-Damluji *et al*., 1993). Displacement of [^{3}H]-prazosin by unlabelled prazosin is due to the presence of alpha-1 adrenoceptors (Al-Damluji *et al*., 2001) and the paradoxical increase in accumulation of [^{3}H]-prazosin is due to activation of an unusual uptake process designated Transport-P (Al-Damluji & Kopin, 1996a,b).

Uptake processes for amines were known to exist in pre-synaptic nerve terminals and in glial cells (Iversen, 1967). In pre-synaptic nerve terminals, transporter molecules which are located in the plasma membrane utilize the electrochemical gradient of sodium ions which is generated by the Na^+/K^+ATPase to accumulate molecules of neurotransmitter from the extracellular space into the cytoplasm (Amara & Kuhar, 1993; Lester *et al*., 1994). These plasma membrane transporters can be blocked by antidepressants (Glowinski & Axelrod, 1964; Giros *et al*., 1994; Barker *et al*., 1994). Transport-P resembles pre-synaptic plasma membrane transporters in its sensitivity to antidepressants but it differs as

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follows: Transport-P is located in post-synaptic peptidergic neurones (Al-Damluji *et al*., 1997); in pre-synaptic neurones, increasing concentrations of unlabelled neurotransmitter amine do not cause a paradoxical increase in accumulation of the radiolabelled ligand (Al-Damluji & Kopin, 1996a); pre-synaptic re-uptake is linked to a P-ATPase (phosphorylated-type ATPase, the Na^+/K^+ ATPase) whereas Transport-P is linked to V-ATPase (Al-Damluji & Kopin, 1996a); Transport-P is independent of sodium which is required for pre-synaptic plasma membrane uptake (Al-Damluji & Kopin, 1996a); efficacy at Transport-P is reduced by the presence of phenolic hydroxyl groups in the ligands, whereas these phenolic hydroxyl groups increase efficacy at pre-synaptic plasma membrane uptake (Al-Damluji & Kopin, 1998); efficacy at Transport-P is unaffected by the presence of phenolic methoxyl groups in the ligands, whereas these phenolic methoxyl groups reduce efficacy at pre-synaptic plasma membrane uptake (Al-Damluji & Kopin, 1998).

Following uptake into the cytoplasm by pre-synaptic plasma membrane transporters, the amines are accumulated into acidified neurosecretory vesicles by pre-synaptic vesicular transporters (Schuldiner *et al*., 1995; Liu & Edwards, 1997). Transport-P resembles pre-synaptic vesicular transporters in its dependence on protons and V-ATPase but it differs as follows: Transport-P is located in post-synaptic peptidergic neurones (Al-Damluji *et al*., 1997); Transport-P is unaffected by reserpine which inhibits pre-synaptic vesicular transporters (Al-Damluji & Kopin, 1996a); efficacy at Transport-P is reduced by the presence of phenolic hydroxyl groups in the ligands, whereas these phenolic hydroxyl groups increase efficacy at pre-synaptic vesicular uptake (Al-Damluji & Kopin, 1998); Transport-P does not accumulate serotonin or histamine which are accumulated by pre-synaptic vesicular transporters (Al-Damluji & Kopin, 1998); cocaine inhibits Transport-P but has no effect on pre-synaptic vesicular transporters (Al-Damluji & Kopin, 1998).

The uptake process in glial cells ($Uptake_2$) is insensitive to antidepressants and reserpine but is blocked by steroid hormones (Russ *et al*., 1996). Transport-P differs from $Uptake_2$ as follows: Transport-P is located in neurones (Al-Damluji *et al*., 1997); Transport-P is unaffected by steroid hormones which inhibit $Uptake_2$ (Al-Damluji & Kopin, 1996a); $Uptake_2$ is independent of protons which are required for Transport-P (Al-Damluji & Kopin, 1996a); efficacy at Transport-P is unaffected by phenolic methoxyl groups in the ligands, whereas these methoxyl groups increase efficacy at $Uptake_2$ (Al-Damluji & Kopin, 1998); efficacy at Transport-P is enhanced by alpha-methyl and reduced by beta-hydroxyl groups in the ligands, whereas these substitutions have the opposite effect on the efficacy of ligands at $Uptake_2$ (Al-Damluji & Kopin, 1998); Transport-P does not accumulate serotonin, histamine, isoprenaline or MPP^+ which are accumulated by $Uptake_2$ (Al-Damluji & Kopin, 1998).

Previously, we postulated that following uptake by Transport-P, the amines may be recycled and released at another site (Al-Damluji *et al*., 1993). We have now examined this possibility by studying the release of amines following uptake by Transport-P in immortalized gonadotrophin-releasing hormone (GnRH) neurones (GT1-1 cells). These hypothalamic peptidergic neurones retain many properties of differentiated neurones, including expression of neuronal markers and ion channels, and synthesis, storage and secretion of the neurotransmitter GnRH in a pulsatile manner which is characteristic of its secretion *in vivo* (Mellon *et al*., 1990; Krsmanovic *et al*., 1992; Weiner *et al*., 1992; Wetsel *et al*., 1992).

Methods

Cell culture

Immortalised 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 or 175 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% foetal bovine serum (FBS), sodium bicarbonate 3.7 g/l and gentamicin 100 mg/l, 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, deoxyribonuclease I (DNAseI) and ethylenediaminetetraacetic acid (EDTA) and incubated in Corning 12-well plates (2×10^6 cells $well^{-1}$) for studying the release of prazosin, or in Nunc two-well glass chamber slides (10^5 cells $chamber^{-1}$) for studying the release of BODIPY FL prazosin. The culture wells and the glass chamber slides had been coated with poly-D-lysine and laminin. Culture media were changed at 48-h intervals.

Release of prazosin from immortalized GnRH neurones

Release studies were performed on intact cells. GT1-1 GnRH cells were grown in 12-well plates which had been coated with poly-D-lysine (2.5 $\mu g/cm^2$; Sigma P-6407; MW 70,000-150,000) and laminin (0.25 $\mu g/cm^2$; Sigma L-2020). Culture media were changed at 48-h intervals. Drugs were dissolved in uptake/release buffer consisting of DMEM with 25 mM HEPES and 0.5 mM sodium ascorbate, pH 7.4. After 4 days in culture, the cells were washed twice with buffer at 25°C then incubated at 37°C for 60 min in the presence of [^{3}H]-prazosin 2×10^{-9} M and unlabelled prazosin 10^{-6} M. Accumulation of prazosin reaches equilibrium within 60 min (Al-Damluji & Kopin, 1996b). At the end of the incubation period, the buffer was removed and the '0 minute' wells were washed twice with 1 ml buffer at 25°C. Washing the '0 minute' wells occupied less than 30 s. The remaining wells were washed once with 1 ml buffer at 25°C and then incubated in 1 ml buffer with or without the indicated compounds. Unlabelled prazosin was not added to the release buffer, except in the experiment which is described in Figure 2B. After various time intervals, the buffer was removed and the cells were solubilized with 2 ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M sodium hydroxide. Fifty microlitre 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 scintillation spectrometer 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 & Warriner (Chester, Cheshire, U.K.).

Initial studies indicated that desipramine does not inhibit amine release from GnRH cells (Figure 1); subsequent release studies were therefore carried out in the presence of desipramine 10^{-5} M to prevent re-uptake of released prazosin from the incubation medium. In the presence of desipramine, release of prazosin from GnRH cells followed an exponential curve (Figure 1) which fitted the equation $y = k10^{ct}$ where y is the specific cellular content of prazosin at time t, k is the specific cellular content of prazosin at time 0 min, c is the release constant (in seconds^{-1}) and t is time. The release constant c is also equal to the gradient of the regression trendline in the log-linear plots. The half-life of retained cellular prazosin was calculated from these log-linear plots which were obtained using an exponential curve-fitting function in Microsoft Excel on a Macintosh computer. The reliability of a curve fit was assessed using the R^2-value.

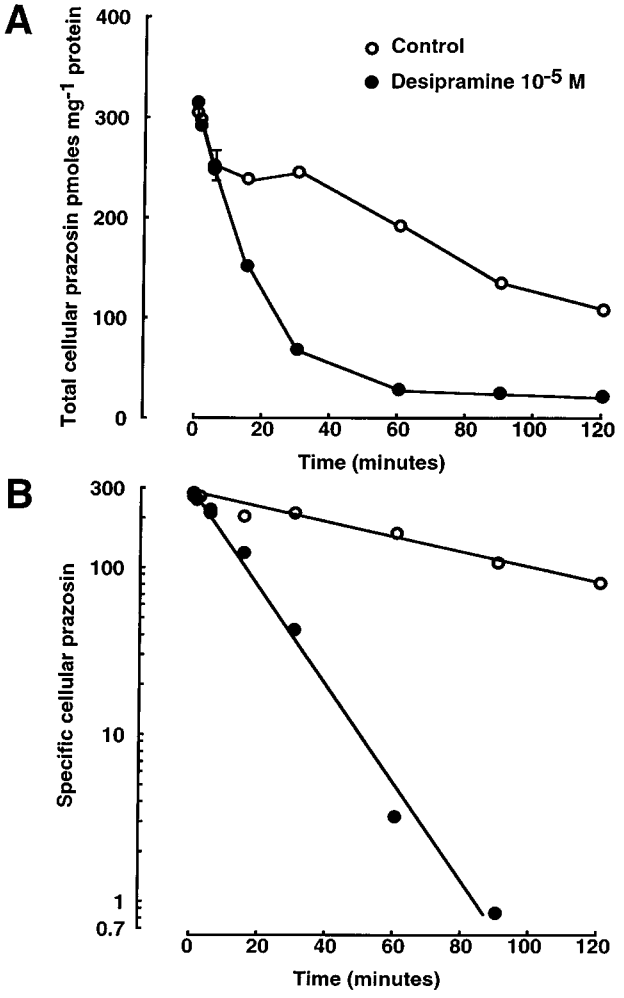

Figure 1 Effect of desipramine on the release of prazosin from GnRH cells following accumulation by Transport-P. The data are presented as linear plots of the total cellular content of prazosin in (A) and as log-linear plots of the specific cellular content of prazosin in (B). Non-specific uptake was defined as the residual amount of cellular prazosin in the presence of desipramine 10^{-5} M at 120 min. Specific content was obtained by subtracting non-specific content from total content. In the presence of desipramine, release of prazosin follows an exponential time course which is described by a straight line in the log-linear plot. The R^2 values for the curve fits in (B) were: control 0.968; desipramine 0.9908. The study was carried out at 37°C. In this and in subsequent figures, units were omitted when data were log-transformed.

The effect of temperature on the release of prazosin was analysed using both linear and Arrhenius plots. In the linear plots, the temperature quotient (Q_{10}) was defined according to Price & Stevens (1989) as the ratio of the release constant at (T + 10K)/TK where T is 300 K (27°C). Q_{10} is also given by the expression $e^{EA/75000}$ where 'e' is the natural logarithm (2.71828) and 'Ea' is the activation energy in kJ mol^{-1} (Price & Stevens, 1989). The activation energy was calculated from the Arrhenius plot in which the inverse of absolute temperature is plotted against the natural logarithm of the release constant (Cornish-Bowden, 1995). In such a plot, the gradient is given in the expression -Ea/R where 'R' is the gas constant (8.31451 Jmol^{-1} K^{-1}).

The data are presented as cellular content of prazosin (labelled and unlabelled) by accounting for the fall in specific activity of [^{3}H]-prazosin consequent upon mixing with unlabelled prazosin, and expressed as pmoles prazosin mg^{-1} protein. These units were omitted when data were log-transformed in the exponential plots. Each experimental point was carried out in triplicate and each experiment was replicated at least once. The minimum number of estimations for each experimental point was therefore six. The data are expressed as the means ± s.e.mean. Standard error bars are not shown where they are smaller than the sizes of the symbols. Statistical comparisons were by analysis of variance (ANOVA; single factor) which was performed using a function on Microsoft Excel. P values less than 0.05 were considered significant.

Release of BODIPY FL prazosin from immortalized GnRH neurones

The fluorescence microscopy method for BODIPY FL prazosin has been described in detail (Al-Damluji *et al.*, 1997). Briefly, GT-1 GnRH cells were grown in 2-well glass chamber slides which had been coated with poly-D-lysine (5 μg/cm^2) and laminin (0.5 μg/cm^2). Cell density at seeding was 25,000 cells cm^2 and other culture conditions were as described above. After 2–3 days in culture, the cells were washed twice with Krebs-Ringer-HEPES (KRH) buffer (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 25; pH 7.4) at 25°C. The cells were then incubated at 37°C for 60 min in the presence of the fluorescent compound BODIPY FL prazosin $1.77X10^{-7}$ M in KRH buffer. The cells were then washed twice with KRH buffer and incubated in the presence of desipramine 10^{-5} M, with or without the indicated compounds in KRH buffer for 15 min. 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 Eclipse E800 fluorescence microscope as previously described (Al-Damluji *et al.*, 1997). All photographs were taken using the X40 objective and Kodak ISO 3200 black and white film which was exposed for 4.26 s.

Materials

Immortalized GnRH neuronal cells (Mellon *et al*., 1990) were generously provided by Dr R.I. Weiner. Heat-inactivated FBS, gentamicin and Nunc glass chamber slides were from Life Technologies (Paisley, U.K.). [^{3}H]-Prazosin was from Amersham (TRK.843; batch 45; specific activity 78 Ci m-mole^{-1}). Unlabelled compounds and culture media were from Sigma-Aldrich (Poole, Dorset, U.K.). BODIPY FL prazosin was from Molecular Probes (Eugene, California, U.S.A.; catalogue number B-7433; lot number 3241-3). This compound was dissolved in dimethylsulphoxide at a concentration of 177 μM and stored in aliquots at -20°C in the dark. Reagents for protein assay were from Pierce & Warriner (Chester, Cheshire, U.K.).

Results

Following uptake by Transport-P, there was a decline in the total cellular content of prazosin which was accelerated by desipramine 10^{-5} M (Figure 1A). Non-specific uptake was defined as the residual amount of cellular prazosin in the presence of desipramine 10^{-5} M at 120 min (Figure 1A). In the presence of desipramine, the decline of the specific cellular content of prazosin was exponential and could be described by a straight line on a log-linear plot (Figure 1B; R^2 value for the curve fit 0.9908; control: release constant 7.3×10^{-5} sec^{-1}, half life 69 min; desipramine 10^{-5} M; release constant 4.7×10^{-4} sec^{-1}, half life 11 min; ANOVA: $P=0.01$). The effect of desipramine was concentration-dependent (IC_{50} 3×10^{-6} M). As desipramine inhibits the uptake of prazosin in these cells (Al-Damluji & Kopin, 1996b), the effect of desipramine on the cellular content of prazosin was interpreted as being due to inhibition of re-uptake of released prazosin from the incubation medium. This hypothesis was tested further by examining the effect of unlabelled prazosin in the release buffer on the cellular content of [^{3}H]-prazosin. One of the functional properties of Transport-P is that at concentrations of unlabelled prazosin greater than 10^{-7} M, there is a paradoxical increase in accumulation of [^{3}H]-prazosin (Al-Damluji *et al*., 1993; Figure 2A). This is due to activation of the uptake process by its substrate (prazosin), resulting in an exponential increase in the accumulation of the substrate. This increase in accumulation of prazosin is antidepressant-blockable (Figure 2A). We tested the hypothesis that released prazosin can be re-accumulated from the medium *via* Transport-P, by examining the effects of unlabelled prazosin 10^{-6} M with and without the antidepressant desipramine in the release buffer. All the cells were pre-loaded with [^{3}H]-prazosin and unlabelled prazosin 10^{-6} M; release was then studied in the absence of drugs (control) or in the presence of unlabelled prazosin 10^{-6} M, desipramine 10^{-5} M or the combination of unlabelled prazosin and desipramine. In the presence of unlabelled prazosin in the release buffer, the cellular content of [^{3}H]-prazosin was increased ($P<0.01$ at 60 min; Figure 2B); this increase was abolished by desipramine (cellular content of [^{3}H]-prazosin at 60 min: control: $15{,}096\pm983$ d.p.m.; unlabelled prazosin 10^{-6} M: $25{,}521\pm493$ d.p.m.; desipramine 10^{-5} M: $4{,}966\pm56$ d.p.m.; unlabelled prazosin + desipramine: $13{,}381\pm675$ d.p.m.). Thus, the cells can accumulate the small amounts of [^{3}H]-prazosin which are present in the release buffer by a mechanism whose functional properties are identical to the uptake process which has been described in these cells (Transport-P). The rate of release of [^{3}H]-prazosin was very similar in cells which were studied in the presence of desipramine + unlabelled prazosin and in the cells which were studied in the presence of desipramine alone in the release buffer (release constant: control 9.9×10^{-5} s^{-1}; unlabelled prazosin 3.5×10^{-5} s^{-1}; desipramine 2.4×10^{-4} s^{-1}; unlabelled prazosin + desipramine 1.4×10^{-4} s^{-1}).

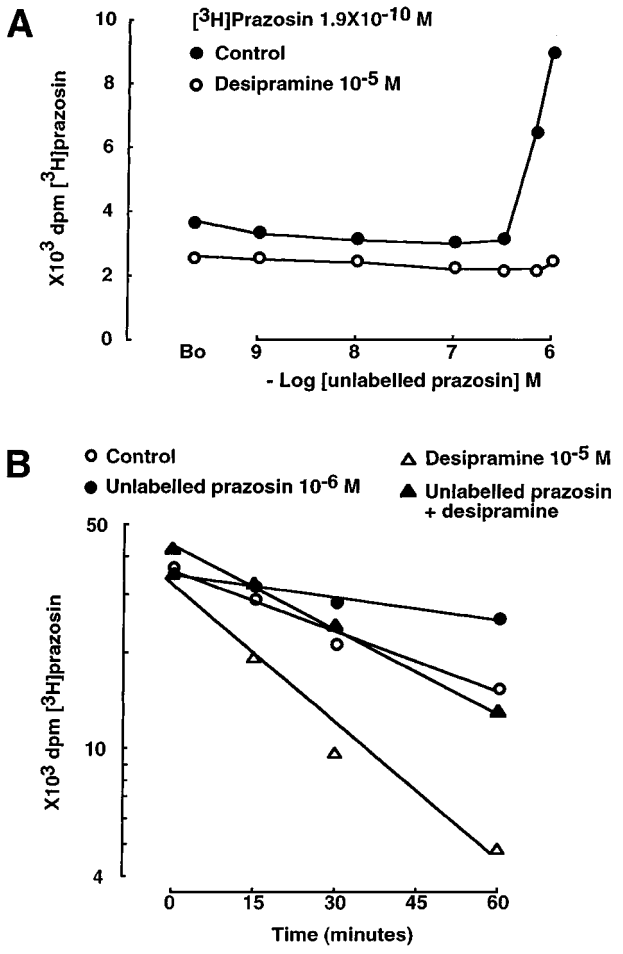

Figure 2 (A) Effect of unlabelled prazosin on the uptake of [^{3}H]-prazosin in GT1-1 GnRH cells. (B) Effect of unlabelled prazosin 10^{-6} M in the release buffer on the cellular content of [^{3}H]-prazosin in GT1-1 GnRH cells. All the cells were pre-loaded with [^{3}H]-prazosin and unlabelled prazosin 10^{-6} M; release was then studied in the absence of drugs (control) or in the presence of unlabelled prazosin 10^{-6} M, desipramine 10^{-5} M or the combination of unlabelled prazosin and desipramine. The R^2 values for the curve fits in (B) were: control 0.9864; unlabelled prazosin 0.9834; desipramine: 0.9281; unlabelled prazosin + desipramine: 0.9993. The experiments in (A) and (B) were performed at 37°C.

In order to avoid interference from re-uptake of released prazosin, further experiments were carried out in the presence of desipramine 10^{-5} M. This concentration of desipramine maximally inhibits the uptake of prazosin in GnRH cells (Al-Damluji & Kopin, 1996b).

In 16 independent experiments in the presence of desipramine 10^{-5} M at 37°C, the coefficient of variation

(CV) of the release constant was 17% and the CV of the half life was 18%.

In the presence of desipramine 10^{-5} M, release of prazosin was acutely sensitive to the temperature of the incubation medium (Figure 3). This is clearly evident when the data in Figure 3 are presented as a linear plot of temperature *vs* release constant (Figure 4A). There was essentially no release of prazosin at 0°C (release constant 3.3×10^{-6} s^{-1}; half life 1510 min). There was a small increase in release at temperatures up to 25°C (release constant 4.2×10^{-5} s^{-1}; half life 120 min). However, at temperatures greater than 25°C, there was a sharp acceleration in release which reached a maximum at 33°C (release constant 2.5×10^{-4} s^{-1}; half life 20 min). There was no further increase in release at 37°C (Figure 4A). The approximate value of Q_{10} calculated from the linear plot was 2.6 (Figure 4A). The data in Figure 3 are also presented as an Arrhenius plot of temperature K^{-1} against the natural log of the release constant (Figure 4B). The data fitted a straight line ($R^2 = 0.9625$) whose gradient was -10^4 K. The gradient is also given in the expression -Ea/R (Cornish-Bowden, 1995). From this, a value of 83.1 kJ mol^{-1} was obtained for the activation energy for the release of prazosin from GnRH cells. Q_{10} is also given by the expression $e^{Ea/75000}$ (Price & Stevens, 1989) from which a value of 3.0 was obtained. This is similar to the approximate Q_{10} value which was obtained from the linear plot.

The organic base chloroquine accelerated prazosin release in the presence of desipramine (Figure 5A; control: release constant 2.5×10^{-4} s^{-1}; half life: 20 min; chloroquine 10^{-4} M: release constant 7.3×10^{-4} s^{-1}; half life 7 min; ANOVA: $P < 0.01$). The effect of chloroquine was concentration-dependent (Figure 5B; IC_{50} 4.6×10^{-5} M).

The ionophore monensin accelerated the release of prazosin in the presence of desipramine at 37°C (Figure 6A; control: release constant 1.9×10^{-4} s^{-1}; half life: 26 min; monensin 10^{-5} M: release constant 5.7×10^{-4} s^{-1}; half life 9 min; ANOVA: $P < 0.01$). The effect of monensin was concentration-dependent (Figure 6B; IC_{50} 3.8×10^{-6} M).

In the presence of desipramine 10^{-5} M at 37°C, release of prazosin was accelerated by the V-ATPase inhibitor bafilomycin A1 (Figure 7; control: release constant 2.1×10^{-4} s^{-1}; half life: 24 min; bafilomycinA1 10^{-6} M; release constant 4.5×10^{-4} s^{-1}; half life 11 min; ANOVA: $P = 0.01$).

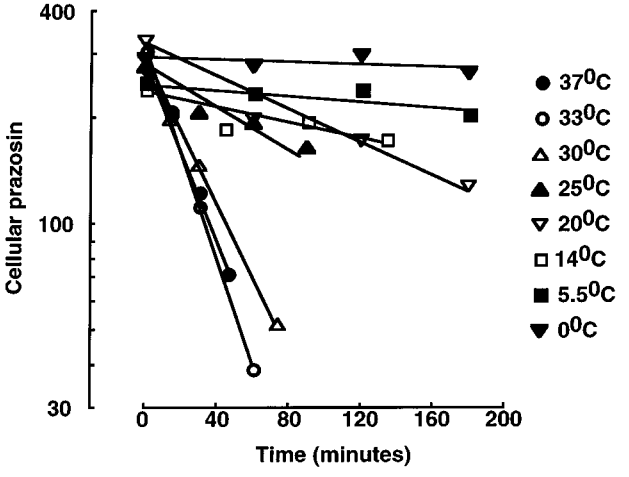

Figure 3 Effect of the incubation temperature on the release of prazosin from GnRH cells: Release was studied at temperatures ranging from 0°C to 37°C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin.

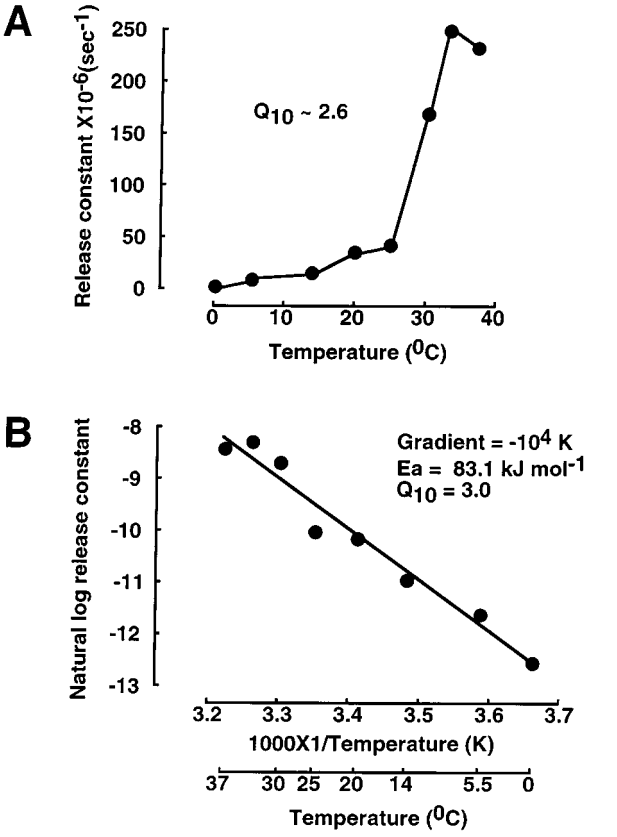

Figure 4 Analysis of the effect of temperature on the release of prazosin from GnRH neurones: In (A) the data from Figure 3 are presented as linear plots of temperature (°C) against the release constants (s^{-1}). In (B) the data from Figure 3 are presented as an Arrhenius plot of temperature K^{-1} against the natural log of the release constant. Increasing temperature has an exponential effect on prazosin release, which is consistent with the Arrhenius equation. Calculations of Ea and Q_{10} are described in the text. The R^2 value for the curve fit in (B) was 0.9625.

Increasing extracellular pH accelerated prazosin release at 37°C in the presence of desipramine 10^{-5} M (Figure 8A; pH 7.4: release constant 1.9×10^{-4} s^{-1}; half life: 27 min; pH 7.7: release constant 3.0×10^{-4} s^{-1}; half life: 17 min; pH 8.3: release constant 4.4×10^{-4} s^{-1}; half life: 11 min; ANOVA: pH 7.4 vs pH 7.7: $P < 0.05$; pH 7.4 vs pH 8.3; $P < 0.001$). In contrast, the release of prazosin was slowed in acidic extracellular pH (Figure 8B; ANOVA pH 7.4 vs pH 6.4: $P < 0.05$).

GT1-1 GnRH cells cultured in the presence of FBS had a predominantly round appearance with few processes (Figure 9), in contrast to the neuronal appearance of these cells when grown in serum-free medium (Al-Damluji *et al.*, 1997). Autofluorescence of GnRH cells (ie, fluorescence in the absence of BODIPY FL prazosin) was minimal, confirming previous observations (Al-Damluji *et al.*, 1997). GnRH cells incubated at 37°C in the presence of the fluorescent analogue BODIPY FL prazosin accumulated the fluorescent com-

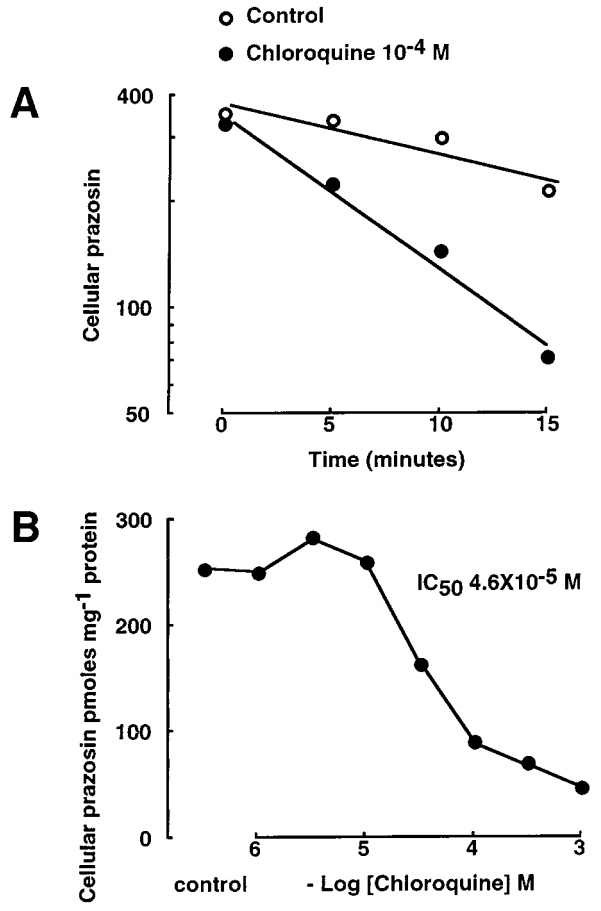

Figure 5 Effect of the organic base chloroquine on prazosin release. Release was studied at 37°C in the presence of desipramine 10^{-5} M. The R^2 values for the curve fits in (A) were: control 0.8901; chloroquine 0.9917. In (B) release was studied at 15 min.

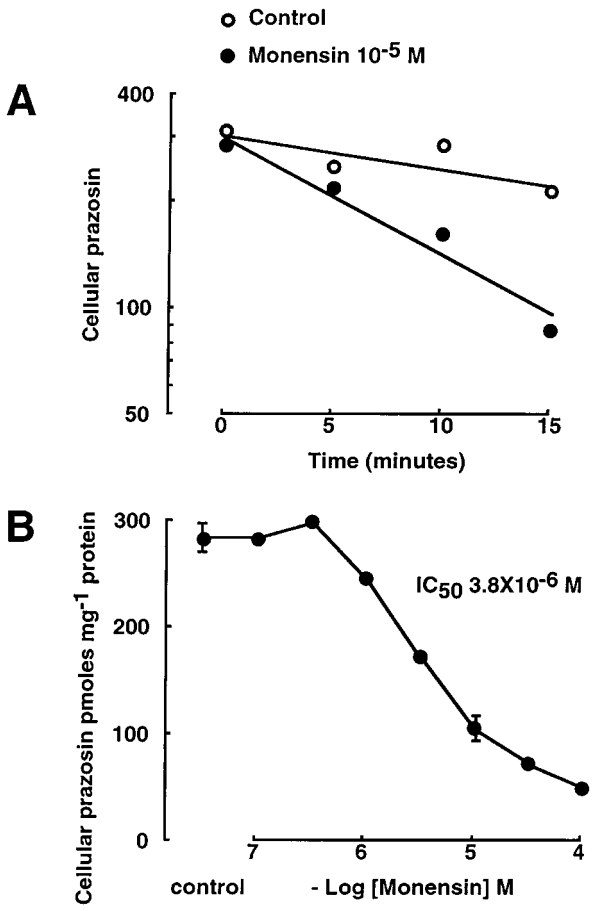

Figure 6 Effect of monensin on prazosin release from GnRH cells: Release was studied at 37°C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin. The R^2 values for the curve fits in (A) were: control 0.7301; monensin 0.9974. In (B) release was studied at 15 min.

pound and became intensely fluorescent. Acquisition of fluorescence was blocked by the antidepressant desipramine 10^{-5} M, confirming that BODIPY FL prazosin accumulates *via* Transport-P, as previously described (Al-Damluji *et al.*, 1997). BODIPY FL prazosin is accumulated *via* Transport-P and it also binds to α_1 adrenoceptors which are located on the cell surface. In the presence of unlabelled prazosin (which blocks the binding of BODIPY FL prazosin to the α_1 adrenoceptors), the nucleus is relatively free of fluorescent staining and the distribution of the fluorescence is in a punctate pattern, as can be expected from accumulation in intracellular vesicles (Figure 9A,B; Al-Damluji *et al.*, 1997). Experiments on the release of BODIPY FL prazosin were carried out in the presence of desipramine 10^{-5} M in order to avoid interference from re-uptake of the released compound. GnRH cells which had accumulated BODIPY FL prazosin at 37°C, washed and incubated in the presence of desipramine 10^{-5} M for 15 min at 37°C remained fluorescent, indicating that some of the fluorescent compound had been retained in the cells (Figure 9C). When release of BODIPY FL prazosin was studied for 15 min at 0°C, the cells retained much more of the fluorescent analogue, as indicated by a more intensely fluorescent appearance of the cells (Figure 9D). In contrast, when release of BODIPY FL prazosin was studied for 15 min at 37°C in the presence of chloroquine 10^{-4} M or monensin 10^{-5} M, cellular fluorescence was greatly diminished (Figure 9E,F), indicating that release of the fluorescent compound had been accelerated.

Discussion

In previous work, we had demonstrated that peptidergic neurones accumulate the amines prazosin and BODIPY FL prazosin *via* Transport-P. The present study demonstrates that these cells are also capable of releasing the accumulated amines. In addition to accumulation by Transport-P, GnRH neurones possess alpha-1B adrenergic receptors which also bind [^{3}H]-prazosin (Al-Damluji *et al.*, 2001). However, the present study examined the release of [^{3}H]-prazosin which had been accumulated in the presence of unlabelled prazosin 10^{-6} M; this concentration of unlabelled prazosin completely blocks the binding of [^{3}H]-prazosin to alpha-1B adrenergic receptors (Al-Damluji & Kopin, 1996a). The decline of cellular radioactivity therefore represents release of prazosin accumulated by Transport-P, rather than dissociation of [^{3}H]-prazosin from alpha-1B adrenergic receptors in GnRH neurones.

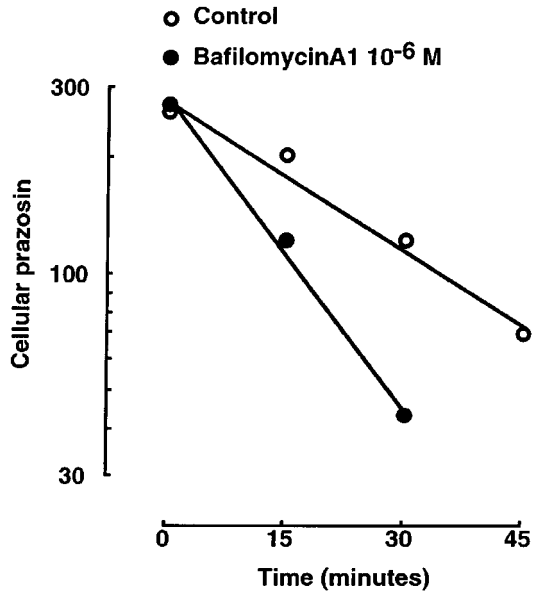

Figure 7 Effect of the V-ATPase inhibitor bafilomycinA1 on prazosin release from GnRH cells: Release was studied at 37°C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin. The R^2 values for the curve fits were: control 0.9935; bafilomycin 0.9951.

Many transporters are capable of reversing the direction of transport if the concentrations of their substrates are reversed; this applies to the pre-synaptic transporters for noradrenaline, dopamine and serotonin and for the $Uptake_2$ carrier in non-neuronal cells (Paton, 1973; Raiteri *et al*., 1977; 1979; Grohmann, 1988). These transporter-mediated release processes are inhibited by appropriate blocking agents such as desipramine, cocaine, nomifensine or O-methyl-isoprenaline which inhibit the uptake of these amines *via* the transporters (Paton, 1973; Raiteri *et al*., 1979; Grohmann, 1988). However, release of prazosin and BODIPY FL prazosin was not blocked by desipramine 10^{-5} M. As this concentration of desipramine completely blocks Transport-P (Al-Damluji & Kopin, 1996b), the findings indicate that release of prazosin occurs by a mechanism other than Transport-P.

In fact, desipramine accelerated the decline of the cellular content of prazosin (Figure 1), presumably by inhibiting re-uptake of released prazosin from the incubation medium. This interpretation is supported by the finding that the presence of unlabelled prazosin in the release buffer increased the cellular content of [^{3}H]-prazosin by an antidepressant-sensitive process (Figure 2B); these findings are identical to the functional properties of Transport-P which is activated by concentrations of prazosin greater than 10^{-7} M (Al-Damluji *et al*., 1993; Figure 2A). Thus, the cells can accumulate the small amounts of [^{3}H]-prazosin which are present in the release buffer *via* Transport-P. It therefore appears that in the control experiment in Figure 1A, the decline in the cellular content of prazosin is not truly mono-exponential, as it represents a composite of the release of cellular prazosin and re-uptake of released prazosin. However, when the re-uptake is inhibited by the presence of desipramine in the release buffer, the decline in the cellular content of prazosin is probably truly mono-exponential.

An alternative interpretation of the effect of desipramine is that it may exert its effect at an intracellular site such as the vesicles which accumulate prazosin. Exit of prazosin from the cells may be by a two-stage process: exit across the vesicular membrane followed by exit across the plasma membrane. It is possible that desipramine may block re-uptake of prazosin from the cytoplasm into the vesicles; accumulation of prazosin in the cytoplasm would then accelerate its exit from the cells. In the present study, we have described some of the functional properties of the release of amines from peptidergic neurones but we have not identified the rate limiting step which is likely to be the site of action of the experimental manipulations.

Release of amines from GnRH cells was acutely sensitive to temperature. Release was minimal below 30°C and accelerated sharply at higher temperatures. The kinetics of temperature dependence conformed to the Arrhenius equation in which there is an exponential increase in reaction rate with temperature (Figure 4; Price & Stevens, 1989). This is typical of cellular processes such as enzymatic reactions (Cornish-Bowden, 1995). In contrast, simple diffusion along a concentration gradient increases linearly with temperature, as described in the Fick-Einstein and the Stokes-Einstein equations (Stein, 1986). Release of prazosin from GnRH neurones is clearly not simply by a process of passive diffusion down a concentration gradient. In common with other biological systems, release of prazosin from GnRH neurones requires an activation energy. The values which were obtained for the activation energy (Ea) and for the temperature quotient (Q_{10}) were within the range of values which are expected in a biological system (Price & Stevens, 1989). An alternative explanation for the temperature dependence of the release process is that cellular phospholipid membranes may undergo a 'melting' process which may influence the release of amines; such a possibility cannot be excluded by the available data and will have to be addressed in future studies.

As Transport-P derives its energy from the electrochemical proton gradient (Al-Damluji & Kopin, 1996a), we examined the effects of manipulations of intracellular acidity on the release of prazosin and BODIPY FL prazosin. Chloroquine is an organic base which diffuses into intracellular acidified particles; at a concentration of 10^{-4} M, chloroquine increased the pH of intracellular acidified vesicles in macrophages from approximately 4.7 to 6.4 (Ohkuma & Poole, 1978). 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). At a concentration of 6×10^{-6} M, monensin increased the pH of intracellular acidified vesicles in fibroblasts from 5.0 to 6.2 (Maxfield, 1982). BafilomycinA1 is an inhibitor of V-ATPase proton pumps which are responsible for generating intracellular acidity (Bowman *et al*., 1988). BafilomycinA1 10^{-6} M increased the pH of lysosomes of cultured cells from 5.1 to 6.3 (Yoshimori *et al*., 1991). These compounds increase intracellular pH by different mechanisms. Uptake of prazosin and BODIPY FL prazosin *via* Transport-P is blocked by all these pharmacological manipulations and by increasing extracellular pH (Al-Damluji & Kopin, 1996a; Al-Damluji *et al*., 1997). In contrast, release of prazosin and BODIPY FL prazosin was

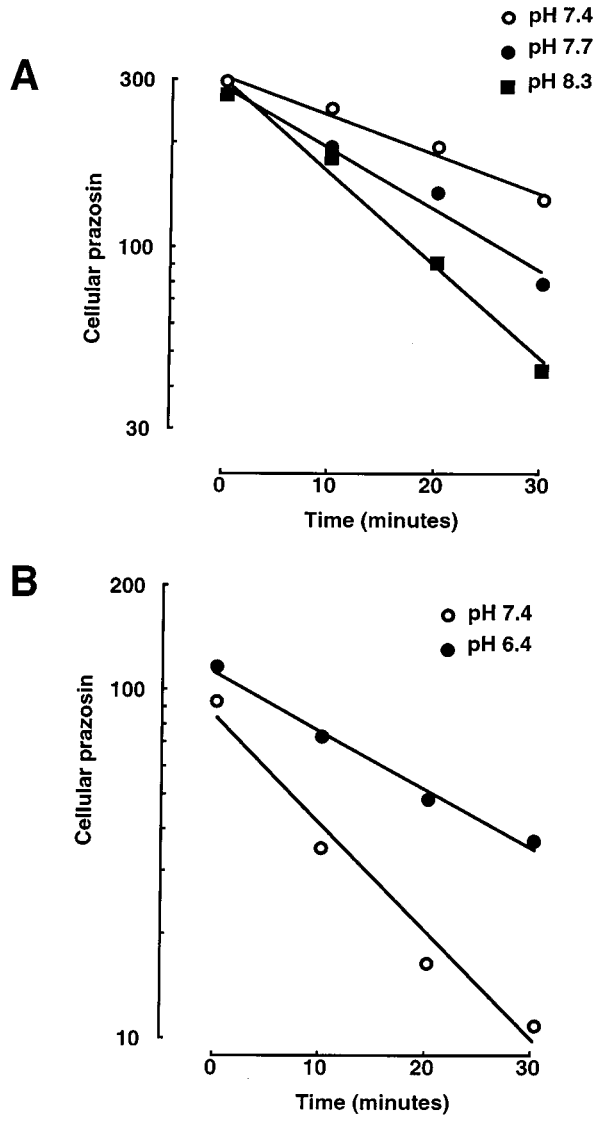

Figure 8 Effect of extracellular pH on prazosin release from GnRH cells: Increasing extracellular pH in the range 7.4-8.3 accelerated prazosin release in the presence of desipramine 10^{-5} M at 37°C (A). Conversely, release was slower in acidic extracellular pH (B). In (A) the R^2 values for the curve fits were: pH 7.4: 0.9835; pH 7.7: 0.9755; pH 8.3: 0.9873. In (B) the R^2 values were: pH 7.4: 0.9703; pH 6.4: 0.9879.

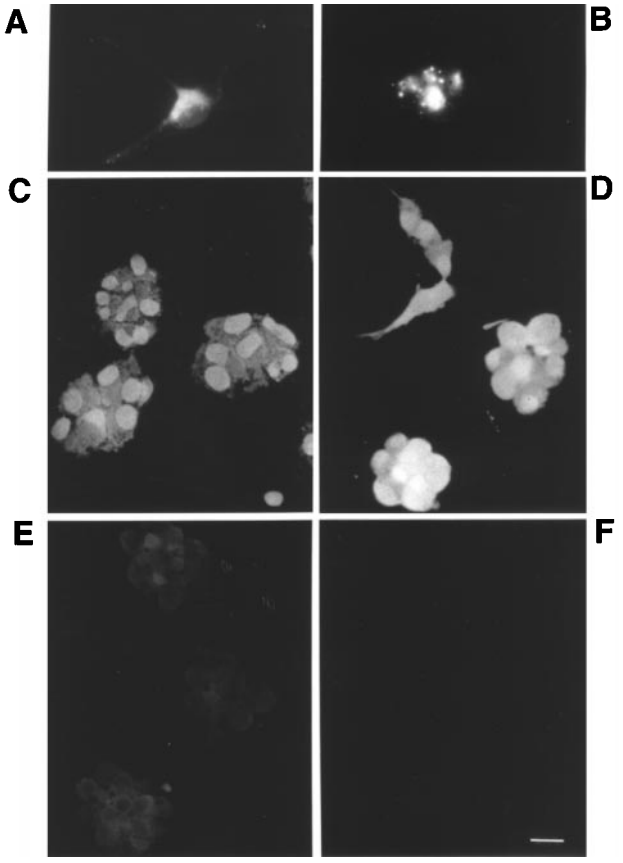

Figure 9 (A,B) Uptake of BODIPY FL prazosin: The fluorescent analogue BODIPY FL prazosin accumulates in GnRH cells *via* Transport-P and it also binds to alpha-1 adrenoceptors on the cell surface. In (A,B) the cells were incubated in the presence of BODIPY FL prazosin and unlabelled prazosin 10^{-6} M (which blocks the binding of BODIPY FL prazosin to the alpha-1 adrenoceptors). Accumulation of BODIPY FL prazosin was then observed after 60 min. The nucleus is relatively free of fluorescent staining (A) and the distribution of the fluorescence is in a punctate pattern (A,B), as can be expected from accumulation in intracellular vesicles (Figure 9B is reproduced from Al-Damluji *et al*., 1997). GnRH cells cultured in the presence of FBS have a predominantly round appearance with few processes, in contrast to the neuronal appearance of these cells when grown in serum-free medium (Al-Damluji *et al*., 1997). C-F: Release of BODIPY FL prazosin: In these studies, the cells were exposed to BODIPY FL prazosin for 60 min at 37°C then washed and incubated for 15 min at 37°C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released BODIPY FL prazosin. After 15 min at 37°C, the cells remained fluorescent, indicating that some of the compound had been retained in the cells (C). Release of BODIPY FL prazosin was slowed at 0°C (D), and accelerated by chloroquine (10^{-4} M at 37°C; (E)) and by monensin (10^{-5} M at 37°C; (F)). The scale bar in panel F represents 5 micrometres.

not blocked by any of these pharmacological manipulations or by increasing extracellular pH. These findings further distinguish the release process from Transport-P.

In fact, release of prazosin was accelerated by these pharmacological manipulations and by increasing extracellular pH. These experiments were carried out in the presence of desipramine 10^{-5} M which completely inhibits uptake of amines *via* Transport-P (Al-Damluji & Kopin, 1996b). Therefore, the accelerated decline of cellular radioactivity which was caused by these manipulations is not due to inhibition of re-uptake of released amines *via* Transport-P. We concluded previously that Transport-P accumulates amines in acidified intracellular vesicles (Al-Damluji & Kopin, 1996a). The present observation that release of prazosin was accelerated by manipulations which neutralize intracellular acidity is consistent with this conclusion. Clearly, intracellular acidity is required for retention of amines which are accumulated *via* Transport-P in peptidergic neurones.

Lysosomotropic drugs are cationic amphiphilic compounds which exist as bases at physiological pH (pKa approximately 8; De Duve *et al*., 1974). 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 pKa 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 but the organic base chloroquine did not reduce the association of prazosin with COS-7 kidney cells, indicating that prazosin is unlikely to accumulate as a result of lysosomotropic properties (Al-Damluji & Kopin, 1996a). 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). However, uptake of prazosin was in fact inhibited by increasing extracellular pH (Al-Damluji & Kopin, 1996a). It therefore seems unlikely that uptake of prazosin is due to a lysosomotropic effect. Conversely, manipulations of intracellular pH may exert an effect on the equilibrium between neutral and charged forms of basic compounds, and this may influence the retention of these compounds within cells. For a compound whose pKa is 6.8, an increase in the pH of intracellular vesicles from 5.0 to 6.5 would reduce the proportion of the ionized species of the compound from 98.4% to 66.6% (Newton & Kluza, 1978). The neutral species of the compound would then be more likely to escape across cellular membranes.

In conclusion, following uptake by Transport-P in peptidergic neurones, amines are accumulated in acidified intracellular stores; their retention in peptidergic neurones requires maintenance of intracellular acidity. The amines can then be released by a temperature-dependent process which is resistant to antidepressants.

References

ALABASTER, V.A., CAMPBELL, S.F., DANILEWICZ, J.C., GREENGRASS, C.W. & PLEWS, R.M. (1987). 2,4-Diamino-6,7-dimethoxyquinazolines. 2-(4-carbamoylpiperidino) derivatives as α_1-adrenoceptor antagonists and antihypertensive agents. *J. Med. Chem.,* **30,** 999–1003.

AL-DAMLUJI, S. & KOPIN, I.J. (1996a). Functional properties of the uptake of amines in immortalised peptidergic neurones (transport-P). *Br. J. Pharmacol.,* **117,** 111–118.

AL-DAMLUJI, S.& KOPIN, I.J. (1996b). Binding and competitive inhibition of amine uptake at post-synaptic neurones (transport-P) by tricyclic antidepressants. *Br. J. Pharmacol.,* **117,** 811–816.

AL-DAMLUJI, S. & KOPIN, I.J. (1998). Structural properties of phenylethylamine derivatives which inhibit transport-P in peptidergic neurones. *Br. J. Pharmacol.,* **124,** 693–702.

AL-DAMLUJI, S. & KRSMANOVIC, L. (1992). High-affinity uptake of noradrenaline by GnRH cells. *Abs. Endoc. Soc.,* **74,** 197 (abstract).

AL-DAMLUJI, S., KRSMANOVIC, L. & CATT, K.J. (1993). High affinity uptake of noradrenaline in post-synaptic neurones. *Br. J. Pharmacol.,* **109,** 299–307.

AL-DAMLUJI, S., PORTER, D., KRSMANOVIC, L., KNUTSON, J.R. & KOPIN, I.J. (1997). Visual detection of transport-P in peptidergic neurones. *Br. J. Pharmacol.,* **120,** 876–882.

AL-DAMLUJI, S., SHEN, W.B., WHITE, S. & BARNARD, E.A. (2001). α_{1B} adrenergic receptors in gonadotrophin-releasing hormone neurones: relation to Transport-P. *Br. J. Pharmacol.,* **132,** 336–344.

AMARA, S.G. & KUHAR, M.J. (1993). Neurotransmitter transporters: recent progress. *Ann. Rev. Neurosci.,* **16,** 73–93.

BARKER, E.L., KIMMEL, H.L. & BLAKELY, R.D. (1994). Chimeric human and rat serotonin transporters reveal domains involved in recognition of transporter ligands. *Mol. Pharmacol.,* **46,** 799–807.

BOWMAN, E.J., SIEBERS, A. & ALTENDORF, K. (1988). Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Nat. Acad. Sci. U.S.A.,* **85,** 7972–7976.

CORNISH-BOWDEN, A. (1995). Fundamentals of enzyme kinetics. Portland Press, London.

DE DUVE, C., DE BARSY, T., POOLE, B., TROUET, A., TULKENS, P. & VAN HOOF, F. (1974). Lysosomotropic agents. *Biochem. Pharmacol.,* **23,** 2495–2531.

GIROS, B., WANG, Y.M., SUTER, S., MCLESKEY, S.B., PIFL, C. & CARON, M.G. (1994). Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J. Biol. Chem.,* **269,** 15985–15988.

GLOWINSKI, J. & AXELROD, J. (1964). Inhibition of uptake of tritiated-noradrenaline in the intact rat brain by imipramine and structurally related compounds. *Nature,* **204,** 1318–1319.

GROHMANN, M. (1988). The acceleration of the extraneuronal efflux of ^{3}H-(±)-isoprenaline induced by high extracellular potassium. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **337,** 406–407.

IVERSEN, L.L. (1967). The uptake and storage of noradrenaline in sympathetic nerves. Cambridge University Press.

KRSMANOVIC, L.Z., STOJILKOVIC, S.S., MERELLI, F., DUFOUR, S.M., VIRMANI, M.A. & CATT, K.J. (1992). Calcium signaling and episodic secretion of gonadotropin-releasing hormone in hypothalamic neurons. *Proc. Nat. Acad. Sci. U.S.A.,* **89,** 8462–8466.

LEDGER, P.W. & TANZER, M.L. (1984). Monensin – a perturbant of cellular physiology. *Trends Pharmacol. Sci.,* **5,** 313–314.

LESTER, H.A., MAGER, S., QUICK, M.W. & COREY, J.L. (1994). Permeation properties of neurotransmitter transporters. *Ann. Rev. Pharmacol. Toxicol.,* **34,** 219–249.

LIU, Y. & EDWARDS, R.H. (1997). The role of vesicular transport proteins in synaptic transmission and neural degeneration. *Ann. Rev. Neurosci.,* **20,** 125–156.

MAXFIELD, F.R. (1982). Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.,* **95,** 676–681.

MELLON, P.L., WINDLE, J.L., GOLDSMITH, P.C., PADULA, C.A., ROBERTS, J.L. & WEINER, R.I. (1990). Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron,* **5,** 1–10.

NEWTON, D.W. & KLUZA, R.B. (1978). pKa values of medicinal compounds in pharmacy practice. *Drug Intell. Clin. Pharm.,* **12,** 546–554.

OHKUMA, S. & POOLE, B. (1978). Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Nat. Acad. Sci. U.S.A.,* **75,** 3327–3331.

PATON, D.M. (1973). Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit atria. *Br. J. Pharmacol.,* **49,** 614–617.

PRESSMAN, B.C. & FAHIM, M. (1982). Pharmacology and toxicology of the monovalent carboxylic ionophores. *Ann. Rev. Pharmacol. Toxicol.,* **22,** 465–490.

PRICE, N.C. & STEVENS, L. (1989). Fundamentals of enzymology. Oxford University Press.

RAITERI, M., CERRITO, F., CERVONI, A.M. & LEVI, G. (1979). Dopamine can be released by two mechanisms differentially affected by the dopamine transport inhibitor nomifensine. *J. Pharmacol. Exp. Therap.,* **208,** 195–202.

RAITERI, M., DEL CARMINE, R., BERTOLLINI, A. & LEVI, G. (1977). Effect of sympathomimetic amines on the synaptosomal transport of noradrenaline, dopamine and 5-hydroxytryptamine. *European J. Pharmacol.,* **41,** 133–143.

RUSS, H., STAUDT, K., MARTEL, F., GLIESE, M. & SCHOMIG, E. (1996). The extraneuronal transporter for monoamine transmitters exists in cells derived from human central nervous system glia. *European J. Neurosci.,* **8,** 1256–1264.

SCHULDINER, S., SHIRVAN, A. & LINIAL, M. (1995). Vesicular neurotransmitter transporters: from bacteria to humans. *Physiological Rev.,* **75,** 369–392.

SMITH, P.K., KROHN, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analyt. Biochem.,* **150,** 76–85.

STEIN, W.D. (1986). Transport and diffusion across cell membranes. London: Academic Press.

WEINER, R.I., WETSEL, W., GOLDSMITH, P., MARTINEZ DE LA ESCALARA, G., WINDLE, J., PADULA, A., CHOI, A., NEGRO-VILAR, A. & MELLON, P. (1992). Gonadotropin-releasing hormone neuronal cell lines. *Frontiers in Neuroendocrinology,* **13,** 95–119.

WETSEL, W.C., VALENCA, M.M., MERCHENTHALER, I., LIPOSITS, Z., LOPEZ, F.J., WEINER, R.I., MELLON, P.L. & NEGRO-VILAR, A. (1992). Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. *Proc. Nat. Acad. Sci. U.S.A.,* **89,** 4149–4153.

YOSHIMORI, T., YAMAMOTO, A., MORIYAMA, Y., FUTAI, M. & TASHIRO, Y. (1991). Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.,* **266,** 17707–17712.

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