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# THE RELATIONSHIP BETWEEN NERVE TERMINAL ADENOSINE TRIPHOSPHATASES AND NEUROTRANSMITTER RELEASE: AS DETERMINED BY THE USE OF ANTIDEPRESSANT AND OTHER CNS-ACTIVE DRUGS

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**1** The role of adenosine triphosphatases (ATPases) in neurotransmitter release was studied using nerve terminals (synaptosomes) prepared from rat cerebral cortex as a model.

**2** Amitriptyline, nortriptyline, protriptyline, desipramine and imipramine were found to inhibit ATPases at concentrations of $10^{-5}$ M and above. The drugs inhibited both the basal and electrically evoked release of acetylcholine (ACh) and noradrenaline (NA) at concentrations of $10^{-4}$ M and above.

**3** At low concentrations of antidepressants ($10^{-8}$ and $10^{-7}$ M) release of NA was enhanced but there was no effect on ACh release.

**4** Other drugs which inhibit $Na^+$, $K^+$-ATPase increased basal NA release as did drugs which inhibited vesicular $Mg^{2+}$-ATPase.

**5** A model is proposed suggesting that transmitter release/re-uptake depends on (1) active $Na^+$, $K^+$-ATPase at the presynaptic membrane and (2) an active synaptic vesicular $Mg^{2+}$-ATPase.

## Introduction

Tricyclic antidepressant drugs in low concentrations are known to increase the apparent release of noradrenaline (NA) from some tissue preparations *in vitro*, probably by an effect on re-uptake processes (Iversen, 1975). Acetylcholine (ACh) release is not influenced at these concentrations. In contrast, high concentrations of the antidepressants inhibit the release of both ACh and NA from nerve endings (synaptosomes) prepared from the central nervous system (Gilbert, Allen, Townsend & Wyllie, 1978a). As the molecular mechanisms involved in neurotransmitter release and re-uptake are unclear, these effects of the drugs have not yet been satisfactorily explained.

There is substantial evidence that adenosine triphosphatases (ATPases) may be directly or indirectly involved in neurotransmitter release at a variety of synapses (Paton, Vizi & Aboo Zar, 1971; Kirshner, 1975; Matthaei, Lentzen & Philippu, 1976). Tricyclic antidepressants inhibit a number of ATPase enzymes (Caratsch & Waser, 1973; Nag & Ghosh, 1973; Gilbert *et al.*, 1978a), and in this study we have used some clinically established tricyclic antidepressants, as well as a potential new antidepressant, the dibenzocycloheptene derivative (DBC) to determine whether there is a common effect on the ATPases which could be involved in the actions of the drugs on transmitter release from synaptosomes. The synaptosome is made up of membranes, mitochondria and vesicles and each of the components exhibits a number of ATPase activities. The complexity of the possible relationships between these enzymes is such that it has been necessary to employ here drugs other than antidepressants in attempts to clarify the functions of the individual enzymes. We have therefore included experiments with phenytoin, ouabain and the mild CNS stimulant, pemoline.

## Methods

Male, albino Sprague-Dawley rats (200 to 400 g) were used.

Synaptosomes and their components were prepared from the cerebral cortex, by slight modifications of the techniques of Gray & Whittaker (1962) and Whittaker, Michaelson & Kirkland (1964) as described in detail by Gilbert & Wyllie (1976). The fractions were either stored at −23°C before ATPase assay or used immediately for neurotransmitter studies.

For ATPase assays the tissue fractions were resuspended in 50 mM imidazole/HCl buffer pH 7.40 and 0.2 ml samples, each containing approximately 0.2 mg protein were added to buffered media. The media contained (mM): NaCl 150, KCl 10 and $MgCl_2$ 5 for 'total'-ATPase; $MgCl_2$ 5 for $Mg^{2+}$-ATPase; $CaCl_2$ 5 for $Ca^{2+}$-ATPase; $MgCl_2$ 5 and NaCl 150 for $Na^+$-

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ATPase assay. The mixtures (final volume 0.9 ml) were pre-incubated at 37°C for 15 min before the reactions were started by addition of 0.1 ml Tris-ATP solution (4 mM final concentration). Sodium dodecyl sulphate (DDS, 1 ml, 0.8% w/v in distilled water) was used to stop the reaction after 10 min. The phosphate contents of the resulting clear solutions were determined by the method of Bonting, Simon & Hawkins (1961). Blanks were prepared by the addition of DDS before the addition of ATP.

$Na^+$, $K^+$-ATPase activity was calculated by subtracting the $Na^+$-ATPase activity from the 'total'-ATPase activity. $Na^+$-ATPase activity was calculated by subtracting the $Mg^{2+}$-ATPase activity from the activity obtained in the presence of $MgCl_2$ and NaCl together. $Mg^{2+}$-ATPase and $Ca^{2+}$-ATPase were calculated by subtracting the blanks from the activity in the presence of $MgCl_2$ and $CaCl_2$ respectively. When used, drugs were added to the preincubation media. The protein contents of solutions were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Synaptosomal release studies*

Neurotransmitter release studies were carried out in the apparatus shown in Figure 1 (Glass Appliances, Holburn St., Aberdeen). The apparatus consisted of an upper central chamber (A) the base of which was a rough glass sinter (B). The upper portion was surrounded by a water jacket (C-D). A lower chamber was separated from (A) by a glass membrane (G).

Filters were cut with cork borers or a punch and die from sheets of Whatman's No. 42 or 542 filter paper to give an exact fit at the base of A immediately above the supporting glass sinter (B).

*Preparation of samples* Two layers of filter paper cut to give an exact fit were placed on top of the glass sinter. The synaptosome preparation previously adjusted to 400 mM final osmolarity by the slow addition of 0.2 M sucrose was mixed with an equal volume of incubation medium (described below) and 2 ml of mixture was layered onto the filters. This was effected by applying gentle suction at E. The material passing through was stored at −23°C for analysis. The pore size of the filter paper (0.4 to 1.2 μm) was such that most of the synaptosomes were retained on the filters.

A small volume (2 or 3 ml) of the requisite medium for the experiment was added to the synaptosomes. The standard medium employed was a modified Locke solution of the following composition (mM): NaCl 153.5, $MgSO_4$ 2.8, $CaCl_2$ 2.1, KCl 5.65, $NaHCO_3$ 1.8, sucrose 64.3, glucose 8.3 and physostigmine 0.02. When necessary the medium was adjusted to pH 7.40 with imidazole or HCl. In some experiments KCl was added to give a final potassium concentration of 55.65 mM. The medium was gassed with air and the temperature controlled by the thermostated water jacket C-D.

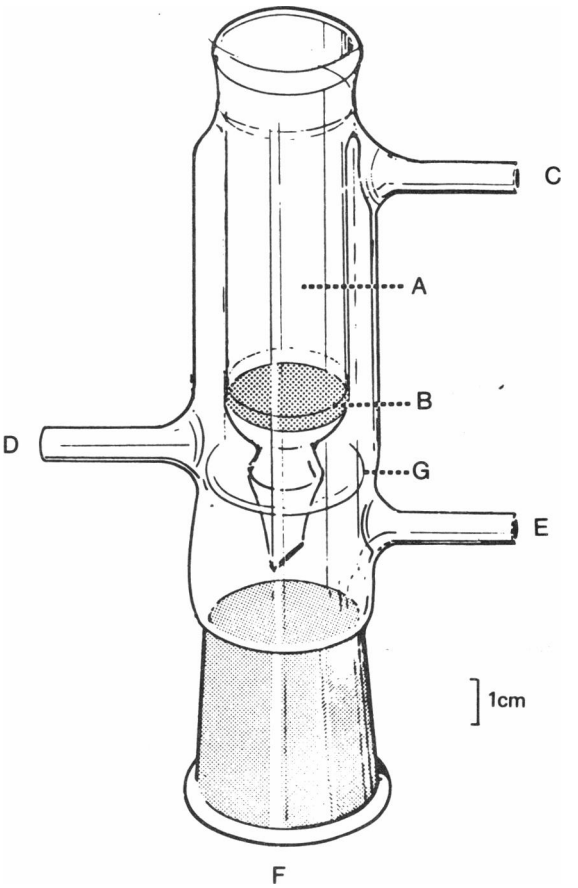


**Figure 1** Apparatus used for synaptosome transmitter release studies. (A) = centre well; (B) = glass sinter; (C–D) = inlet and outlet of water jacket; (E) is connected to suction and the lower chamber is evacuated after sealing aperture (F); (G) is a glass membrane separating the upper and lower chambers. The bar shown is equivalent to 1 cm.

At any desired time the incubation medium was removed by suction at point E after insertion of a suitable container in the lower chamber and sealing aperture F. The filters were quickly exposed to fresh medium or replaced with new filters and the procedure repeated. Filters and samples were stored after use at −23°C for analysis.

Facilities for electrical stimulation of the synaptosomes were included in the circuit when necessary by incorporating a perforated platinum foil disc (elec-

trode to ground) between the supporting glass sinter (B) and the synaptosome-supporting filters. This was then connected into an electrical circuit by a polythene-coated platinum wire. Stimulation was achieved by lowering another coiled platinum wire electrode (live electrode) into the chamber and stimulating with a Farnell Physiological Stimulator. Stimulator output, that is frequency, pulse duration and voltage (or current), was continuously monitored on an oscilloscope. The platinum foil disc was cut for an exact internal fit in chamber A in a similar manner to the Whatman filter paper. Biphasic pulses were employed. Successive pulses were of equal duration but opposite polarity.

NA was assayed fluorimetrically by the technique of O'Hanlon, Campuzano & Horvath (1970). ACh was bioassayed on the superfused leech dorsal muscle. For ACh estimations, all sucrose solutions contained physostigmine (0.02 mM).

The following drugs were used: (DBC) 3-(1a,10b-dihydro-1H-dibenzo (a, e) cyclopropa (c) cycloheptene-6-ylidine)-1-propanamine hydrochloride and SC13504 (1-(2-(6-methylpyridyl)-methylenamino)-4-diphenylmethylpiperazine (G.D. Searle & Co. Ltd.); amitriptyline and protriptyline (Merck, Sharpe & Dohme); nortriptyline (Lilly); phenytoin (Parke-Davis); pemoline (Medo-Chemicals Ltd.); ouabain, (BDH); and imipramine and desipramine (Geigy).

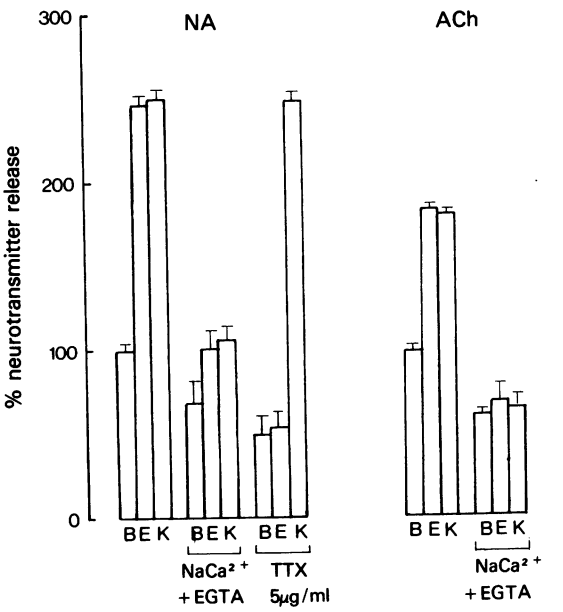


**Figure 2** Effects of different conditions on noradrenaline (NA) and acetylcholine (ACh) release over a 30 min period. Release is expressed as a percentage of basal release. All results are compensated for the release at time zero. Histograms represent the mean of 2 to 6 experiments; vertical lines show s.e. mean. B = basal release; E = electrically evoked release (10 V, 100 Hz, 1 ms); K = $K^+$-induced release (55.6 mM); TTX = tetrodotoxin 5 μg/ml; EGTA = ethylenglycolbis (β-aminoethyl ether) N,N'tetra-acetic acid (1 mM).

## Results

### *Release of acetylcholine and noradrenaline*

The release of ACh and NA from synaptosomes under basal conditions was linear between approximately 5 and 40 min of incubation after which time the rate fell. Under electrically-evoked release conditions the durations of the linear portions of the curves were similar but there were increases in the absolute amounts of the two transmitters released. In one set of experiments, for instance, the amounts of neurotransmitter released under basal and electrically evoked conditions were respectively: 31.2 ± 2.8 and 53.8 ± 2.3 ng ACh $mg^{-1}$ protein $h^{-1}$ (8 experiments) and 27.6 ± 3.4 and 64.5 ± 3.1 ng NA $mg^{-1}$ protein $h^{-1}$ (11 experiments). These values were calculated from the linear portion of the curves between the 5 and 35 min points and this 30 min collection period

**Table 1** Effects of antidepressants on synaptosome ATPases

| *Drug* | *($ED_{50}$ (μM) for ATPase inhibition)* $Na^+, K^+$ | $Na^+$ | $Mg^{2+}$ | $Ca^{2+}$ |
|---|---|---|---|---|
| Amitriptyline | 0.36 ± 0.04 | 0.35 ± 0.08 | 0.29 ± 0.03 | 0.29 ± 0.06 |
| Nortriptyline | 0.53 ± 0.11 | 0.46 ± 0.09 | 0.42 ± 0.09 | 0.41 ± 0.07 |
| Protriptyline | 0.24 ± 0.06 | 0.53 ± 0.14 | 0.43 ± 0.11 | 0.34 ± 0.05 |
| Desipramine | 0.42 ± 0.12 | 0.31 ± 0.09 | 0.39 ± 0.06 | 0.47 ± 0.09 |
| Imipramine | 0.47 ± 0.15 | 0.57 ± 0.21 | 0.57 ± 0.04 | 0.53 ± 0.08 |
| DBC | 0.12 ± 0.04 | 0.18 ± 0.04 | 0.15 ± 0.03 | 0.25 ± 0.04 |

Means and s.e. mean of six or more experiments.

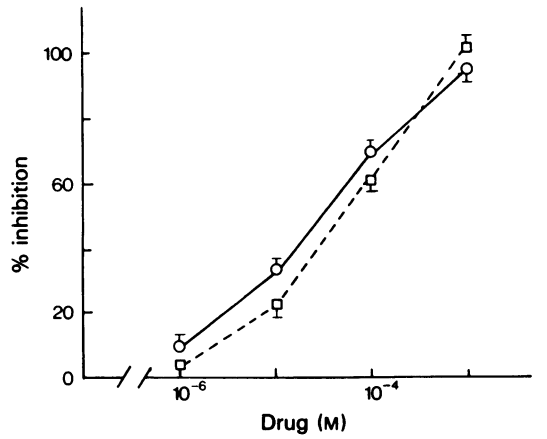


**Figure 3** Dose-response curves of the inhibition of synaptosomal ATPases by protriptyline: (○) inhibition of $Na^+$, $K^+$-ATPase; (□) inhibition of $Mg^{2+}$-ATPase. Results are means of five experiments; vertical lines show s.e. means.

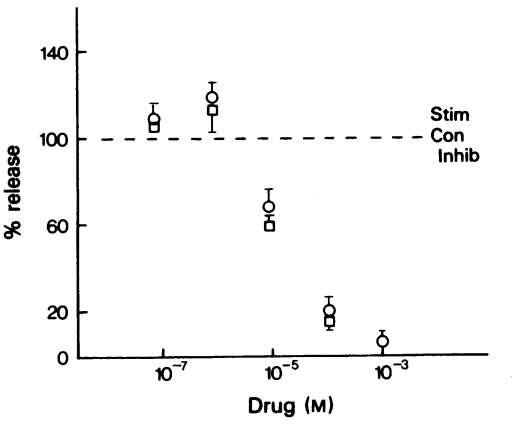


**Figure 4** Effects of protriptyline (○) and DBC (□) on the release of inorganic phosphate from synaptosomes. The composition of the medium (mM) was: NaCl 153.5, $MgSO_4$ 2.8, $CaCl_2$ 2.1, KCl 5.65, $NaHCO_3$ 1.8, sucrose 64.3, glucose 8.3 and physostigmine 0.02. The medium was adjusted to pH 7.40. The phosphate release is compared to the release in the absence of drug (Con) which was taken as 100%. Each point represents the mean of 3 to 7 experiments; vertical lines show s.e. means. Significant stimulation, $P < 0.025$ of inorganic phosphate release by protriptyline at $10^{-6}$ M.

was used in subsequent experiments. In this series then, electrical stimulation evoked a 72% increase in ACh release and 134% increase in NA release.

The evoked release of either ACh or NA was dependent on the frequency of stimulation, voltage and duration of the stimulus. Synaptosomes were capable of evoked release up to 5 h after preparation.

Under supramaximal electrical stimulation conditions (10 V, 100 Hz, 1.0 ms) there was a significant (50 to 150%) increase in neurotransmitter release (Figure 2). A similar or greater degree of increased release was obtained by depolarizing the synaptosomes by the addition of $K^+$ (55.6 mM) or veratridine ($7.5 \times 10^{-5}$ M) (Gilbert, Davison & Wyllie, 1978b). These increments were markedly reduced when $Ca^{2+}$ was omitted from the incubation medium and there was negligible evoked release in a medium lacking $Ca^{2+}$ and containing EGTA (1 mM). Basal release was also reduced when $Ca^{2+}$ was omitted from the incubation medium. Both basal and electrically evoked release were reduced when tetrodotoxin (TTX 5 μg/ml) was added to the incubation medium.

*Effects of antidepressants on ATPases of synaptosomes*

Table 1 shows the $ED_{50}$ values for inhibition of ATPases in the synaptosome fraction by tricyclic antidepressants and DBC. All of the ATPases of the fraction were inhibited by each of the drugs and the amounts of enzyme activity remaining in the fractions at concentrations of drugs giving maximum inhibition were similar (0 to 16% of the control). The degree of inhibition was dependent on the concentration of tricyclic antidepressants (Figure 3). With the exception of DBC which was always the most potent inhibitor, no consistent order of potency of the drugs was apparent when the degrees of inhibition of the individual ATPases were compared. In one series of experiments the release of inorganic phosphate from synaptosomes was measured in a medium of composition such that $Na^+K^+$-, $Na^+$-, $Mg^{2+}$- and $Ca^{2+}$-ATPases, as well as other phosphatase enzymes, were likely to be active. The results (Figure 4) showed that protriptyline, in contrast to the results mentioned above, could significantly increase the activity of one or more enzymes at low concentration ($1 \times 10^{-6}$ M). At this concentration it did not significantly influence the activity of $Na^+K^+$-, $Na^+$-, $Mg^{2+}$- or $Ca^{2+}$-ATPases under the conditions considered most favourable for determining the activities of each enzyme alone.

*Effects of antidepressants on ATPases of synaptosome components*

The effects of protriptyline and DBC on the various ATPases of the synaptosomal components, separated one from another, is shown in Table 2. A much lower concentration of drug was sufficient to give a particular degree of inhibition of any enzyme in the

synaptosomal membrane when compared with that same enzyme in the synaptosome itself (Table 1). In every case DBC was more potent than protriptyline. The vesicles were unique in that they displayed only divalent cation-activated ATPase activity. Extremely high concentrations of protriptyline were found to inhibit the enzyme but the maximum degree of inhibition achieved was only 30%.

Although the intrasynaptosomal mitochondria displayed the complete spectrum of enzyme activity, the ATPases of these organelles were less susceptible to antidepressant inhibition than the corresponding enzymes in the membranes. The absolute $Na^+$, $K^+$-ATPase activity of these mitochondria was different from that of the cell mitochondria (10.4 ± 1.7 (9) as compared with 0.4 ± 0.1 (6) μmol Pi $mg^{-1}$ protein $h^{-1}$) as was the degree of inhibition of the enzyme by protriptyline at a concentration of $10^{-6}$ M (98% compared with 14%).

*Effects of antidepressants on neurotransmitter release*

Protriptyline and DBC did not significantly alter the basal or the electrically evoked release of ACh at a concentration at which no ATPase-inhibitory activity was detectable (1 × $10^{-6}$ M; Figure 5). The two drugs were also tested at higher concentrations and they reduced both basal and evoked release. The stimulation parameters employed for the latter were again 100 Hz, 10 V, 1 ms, for 10 min, conditions which in

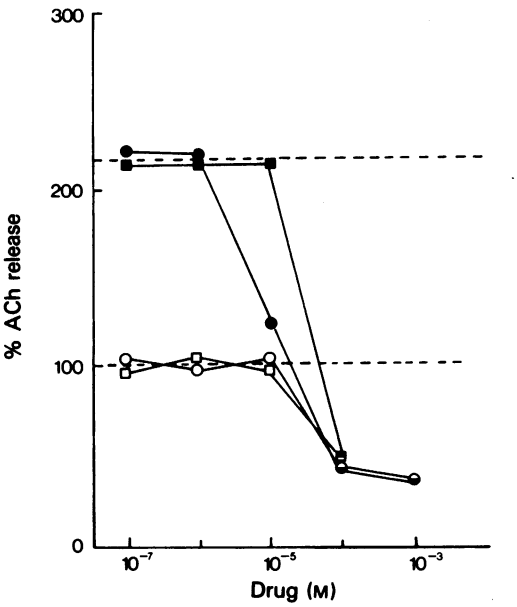


**Figure 5** The effects of protriptyline (○) and DBC (□) on the release of acetylcholine (ACh) from synaptosomes. Open symbols—basal release; closed symbols—stimulated release. Results are means of 6 to 12 experiments and all s.e. means (not shown) are less than 5% of respective means.

**Table 2** Sensitivity of synaptosome ATPases to antidepressants

| *Synaptosomal membranes* | *$ED_{50}$ (μM) for inhibition of synaptosomal ATPases ATPase type* | | | |
|---|---|---|---|---|
| *Drug* | *$Na^+$, $K^+$* | *$Na^+$* | *$Mg^{2+}$* | *$Ca^{2+}$* |
| Amitriptyline | 0.21 ± 0.02 | 0.26 ± 0.08 | 0.24 ± 0.07 | 0.21 ± 0.07 |
| Nortriptyline | 0.32 ± 0.04 | 0.14 ± 0.07 | 0.33 ± 0.08 | 0.31 ± 0.05 |
| Protriptyline | 0.10 ± 0.03 | 0.47 ± 0.09 | 0.33 ± 0.11 | 0.28 ± 0.02 |
| Desipramine | 0.36 ± 0.06 | 0.25 ± 0.04 | 0.31 ± 0.06 | 0.42 ± 0.13 |
| Imipramine | 0.41 ± 0.05 | 0.52 ± 0.16 | 0.56 ± 0.05 | 0.46 ± 0.09 |
| DBC | 0.07 ± 0.02 | 0.08 ± 0.02 | 0.07 ± 0.02 | 0.18 ± 0.04 |
| *Synaptosomal mitochondria* | *$ED_{50}$ (μM) for inhibition of synaptosomal ATPases ATPase type* | | | |
| *Drug* | *$Na^+$, $K^+$* | *$Na^+$* | *$Mg^{2+}$* | *$Ca^{2+}$* |
| Amitriptyline | 1.41 ± 0.28 | 1.51 ± 0.26 | 1.38 ± 0.27 | 1.41 ± 0.16 |
| Nortriptyline | 1.32 ± 0.27 | 1.65 ± 0.31 | 1.52 ± 0.23 | 1.39 ± 0.15 |
| Protriptyline | 1.17 ± 0.23 | 1.29 ± 0.24 | 1.31 ± 0.24 | 1.22 ± 0.13 |
| Desipramine | 1.86 ± 0.25 | 1.91 ± 0.31 | 1.86 ± 0.26 | 1.34 ± 0.29 |
| Imipramine | 1.84 ± 0.28 | 1.68 ± 0.25 | 1.78 ± 0.24 | 1.64 ± 0.27 |
| DBC | 0.91 ± 0.31 | 1.25 ± 0.17 | 0.68 ± 0.09 | 1.03 ± 0.19 |

Mean and s.e. mean of six or more experiments.

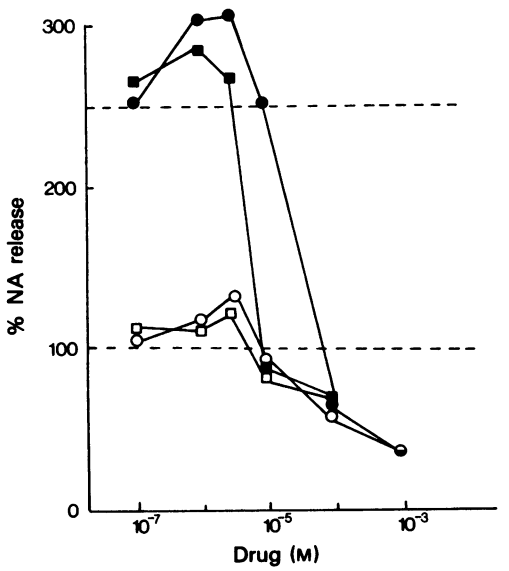


**Figure 6** Effects of protriptyline (○) and DBC (□) on noradrenaline (NA) release from synaptosomes. Open symbols—basal release; closed symbols—stimulated release. Results are the means of 5 to 8 experiments and all s.e. means (not shown) are less than 5% of respective means.

the absence of drugs, usually induced more than 100% increase in release of ACh.

In contrast to the results obtained for ACh, low concentrations of the antidepressants increased the basal release of NA (Figure 6) as found previously (Gilbert *et al.*, 1978a). High concentrations of the drugs ($1 \times 10^{-3}$ and $1 \times 10^{-4}$ M respectively) reduced both the basal and evoked release as in the case of ACh.

*Effects of phenytoin, ouabain and pemoline on neurotransmitter release*

*Acetylcholine* The classical $Na^+$, $K^+$-ATPase inhibitor, ouabain, had no effect on basal ACh release from synaptosomes (Figure 7) and this contrasted sharply with its effect of increasing ACh release from slices by more than 400% (Table 3). Phenytoin, which also inhibits $Na^+$, $K^+$-ATPase likewise failed to alter basal ACh release. Pemoline, which inhibits $Na^+$, $K^+$-ATPase at a concentration of 1 μg/ml but stimulates it at 10 ng/ml (Gilbert *et al.*, 1978a) also had no effect. When the synaptosomes were stimulated electrically both ouabain and phenytoin prevented evoked release and pemoline reduced it significantly at the concentration reducing $Na^+$, $K^+$-ATPase activity.

*Noradrenaline* In marked contrast, basal NA release was significantly increased by all three drugs at the concentrations inhibiting $Na^+$, $K^+$-ATPase (Figure 8). Basal release was inhibited by the concentration of pemoline which stimulated the enzyme. The increase in transmitter release normally associated with electrical stimulation was affected differently by the drugs. Ouabain reduced it markedly, phenytoin reduced it to a smaller extent, while pemoline at the concentration inhibiting $Na^+$, $K^+$-ATPase, reduced it to the same extent as phenytoin but had no effect at the concentration at which it stimulates $Na^+$, $K^+$-ATPase.

*Effect of SC 13504 on transmitter release*

The anticonvulsant SC 13504, which, as far as we can determine, inhibits only the $Mg^{2+}$-ATPase of the vesicles of the ATPases studied here, had no effect on either the basal or the electrically evoked release of ACh (Figure 9). It significantly increased both the basal and evoked release of NA. This effect was apparent at all frequencies of stimulation studied (10, 25 and 100 Hz).

In order to cast more light on possible roles of nerve terminal ATPases in the regulation of transmitter release/uptake, the effects of SC 13504 (vesicular $Mg^{2+}$-ATPase inhibitor), ouabain (membrane $Na^+$, $K^+$-ATPase inhibitor) and phenytoin (membrane $Na^+$, $K^+$-ATPase and vesicular $Mg^{2+}$-ATPase in-

**Table 3** Comparison of the release of acetylcholine from slices and synaptosomes

| | *Synaptosomes* (*ng mg$^{-1}$ protein h$^{-1}$*) | *Cerebral cortex slices* (*ng mg$^{-1}$ protein h$^{-1}$*) |
|---|---|---|
| Basal | 43.6 ± 2.8 (8) (100%) | 21.4 ± 3.6 (8) (100%) |
| Electrically evoked (20 V, 100 Hz, 1 ms) | 154.3 ± 4.3 (6) | 518.2 ± 46.7 (6) |
| Basal + ouabain (0.05 mM) | 99.2 ± 4.1 (4) | 581.7 ± 56.5 (4) |

Results are relative to the basal release, set arbitrarily as 100% and are the means ± s.e. means of 4–8 experiments.

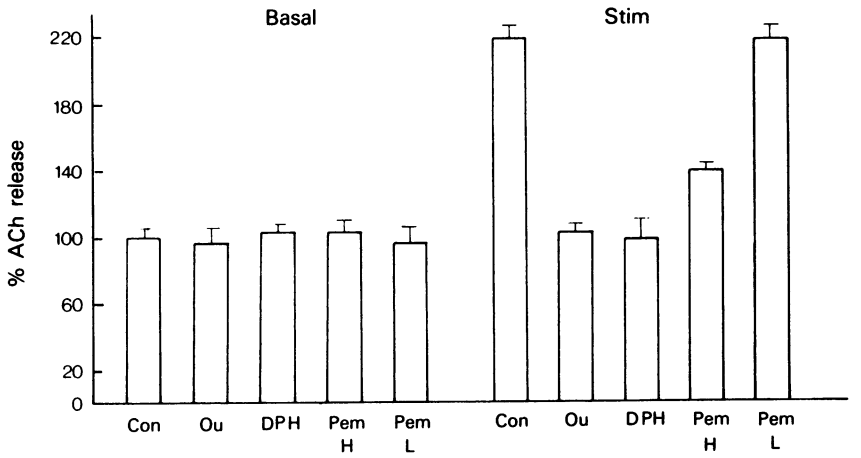


**Figure 7** Effects of drugs on basal and stimulated release of acetylcholine (ACh) from synaptosomes. Scale: the release of ACh is expressed as a percentage ACh release relative to the percentage release in the absence of drugs or electrical stimulation (Basal, Con) and set arbitrarily at 100%. Ou = ouabain 0.1 mM; DPH = phenytoin 0.1 mM; Pem H = pemoline 1 μg/ml; Pem L = pemoline 10 ng/ml; Stim = electrically evoked release (10 V, 100 Hz, 1 ms). All histograms represent the mean of 6 to 15 experiments; vertical lines show s.e. mean.

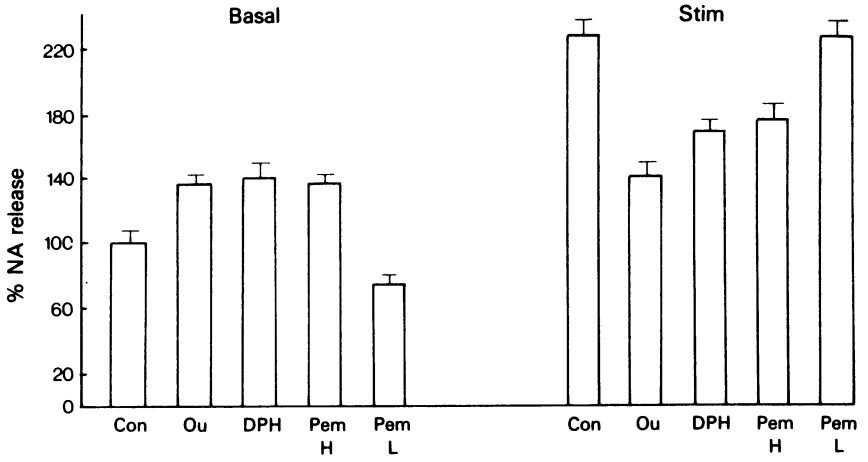


**Figure 8** The effects of drugs on basal and stimulated release of noradrenaline (NA) from synaptosomes. Scale: the release of NA is expressed as a percentage NA release relative to the percentage release in the absence of drugs or electrical stimulation (Basal, Con) and set arbitrarily at 100%. Ou = ouabain 0.1 mM; DPH = phenytoin 0.1 mM; Pem H = pemoline 1 μg/ml: Pem L = pemoline 10 ng/ml: Stim = electrically evoked release (10 V, 100 Hz, 1 ms). All histograms represent the mean of 5 to 12 experiments; vertical lines show s.e. mean.

hibitor) either alone or in combination, were determined on basal and evoked NA release (Table 4). Of the three, only SC 13504 when present alone increased evoked release. The release of NA was significantly reduced by phenytoin and ouabain, only more so in the case of the latter as noted earlier. The addition of SC 13504 had no effect on the reduced release caused by phenytoin. However, if SC 13504 and ouabain were added together, the evoked release of NA was reduced but not to the level attained in the

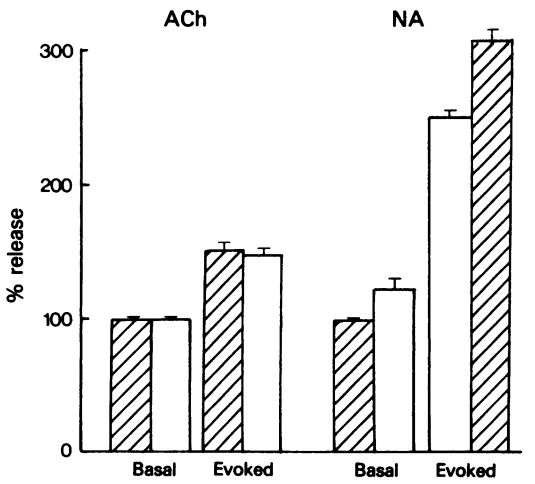


**Figure 9** Effects of SC13504 (0.5 mM) on basal and stimulated release of acetylcholine (ACh) and noradrenaline (NA). Hatched columns = release in absence of drug; open columns = release in presence of drug. Values are means of 6 experiments; vertical lines show s.e. mean.

presence of ouabain. The effect of adding ouabain and phenytoin was not significantly different from the effect of phenytoin alone.

## Discussion

Synaptosome and subsynaptosomal fractions prepared from the CNS by the differential and sucrose density gradient techniques described here have been biochemically and morphologically characterized, and contamination found to be minimal (Gilbert & Wyllie, 1976). In the present work, routine examinations of the preparations have given similar results and we have assumed therefore that our studies reflect effects of conditions on the nerve terminal or its components *in vitro* rather than effects on contaminant material.

All the antidepressants that were tested inhibited the four ATPases of the synaptosome fraction in a dose-dependent manner. This inhibition of synaptosome ATPases is in agreement with the results of Caratsch & Waser (1973) and Nag & Ghosh (1973). Although no one enzyme activity, as determined in the synaptosome fraction as a whole, was generally more sensitive to antidepressant inhibition than others, there was a clear cut difference in the potencies of the drugs and DBC was the most potent inhibitor of each enzyme. This drug was also the most potent with regard to stimulation of NA release.

Studies of the various synaptosomal components separated one from another by sucrose gradient centrifugation following osmotic shock of the synaptosome, revealed that the enzymes located in the synaptosome membrane were most sensitive to antidepressant inhibition. The $ED_{50}$ values of the antidepressants for the enzymes of the intra-synaptosomal mitochondria were approximately three fold higher than the corresponding values for the enzymes of the membranes while the $ED_{50}$ values for the vesicular $Mg^{2+}$-ATPase, the only ATPase with detectable activity in the vesicles under our conditions (Hosie, 1965; Corbett, Christian, Monti & McClain, 1974; Gilbert & Wyllie, 1976), were even higher. This $Mg^{2+}$-ATPase, until now, appeared to be unique in that it was inhibited only by anticonvulsant drugs (Gilbert & Wyllie, 1976). Inhibition of the enzyme by some of the antidepressants in the present work is a new finding but because extremely high concentrations had to be used to obtain an effect this does not detract from our earlier proposal that the enzyme may be a site of action of anticonvulsant drugs at therapeutic concentrations.

The overall effect of the antidepressants on the ATPases of the synaptosome itself appeared to be the net effect of the drugs on the enzymes of the individual components. The different drug sensitivity and enzyme characteristics of the intrasynaptosomal mitochondria, when compared to other synaptosomal components, has been observed with other classes of drugs (Gilbert & Wyllie, 1976). Interestingly, these studies and others (Lai & Clark, 1976; Lai, Walsh,

**Table 4** The additive effects of various drugs on noradrenaline release from synaptosomes

| *Condition* | *% Noradrenaline release* Basal | *Stimulated* |
|---|---|---|
| Control | 100 ± 2.8 (14) | 248.2 ± 3.8 (14) |
| SC13504 (0.5 mM) | 124.5 ± 2.1 (6) | 302.8 ± 4.2 (6) |
| DPH (0.05 mM) | 127.6 ± 3.4 (4) | 201.2 ± 5.1 (4) |
| Ouabain (0.05 mM) | 133.5 ± 2.1 (6) | 141.2 ± 4.5 (4) |
| SC13504 (0.05 mM) + DPH 0.05 mM) | 131.7 ± 2.6 (3) | 198.7 ± 3.4 (3) |
| SC13504 (0.05 mM + ouabain 0.05 mM) | 129.7 ± 2.3 (3) | 197.4 ± 2.3 (3) |
| DPH (0.05 mM + ouabain 0.05 mM) | 130.6 ± 3.4 (3) | 196.8 ± 3.7 (3) |

The values represent the mean and s.e. mean of the number of experiments given in parentheses. Results are relative to release in the absence of drug and electrical stimulation (basal, 100 ± 2.8%).

Dennis & Clark, 1977) have revealed that there are morphological and biochemical differences between mitochondria of synaptic and non-synaptic orgin. This may reflect the more specialized energy requirements of the synapse.

Just how inhibition of these enzymes could account for antidepressant action is a matter for conjecture as are the roles of the enzymes in neurotransmitter release. Involvement of $Na^+$, $K^+$-ATPase in the regulation of NA release has been suggested by Garcia & Kirpekar (1973a, b) who studied the isolated, perfused spleen of the cat. The results with inhibitors of $Na^+$, $K^+$-ATPase (ouabain, phenytoin and pemoline 1 μg/ml) and with pemoline at a concentration which stimulates the enzymes (10 ng/ml) suggest that the basal release of NA is closely linked to the level of $Na^+$, $K^+$-ATPase activity. As the enzyme was inhibited, NA release increased whereas enzyme stimulation resulted in a diminished output. However, it should be noted that under the conditions employed, a steady-state is achieved for any one experimental condition. The concentration of transmitter in the 'release fluid' is the net result of transmitter release, metabolism and re-uptake; alteration of any or all of these could give apparently similar results when these are measured simply as final transmitter concentrations in the fluid. The $Mg^{2+}$-ATPase of the vesicles also appears to play a special role in NA release/re-uptake processes. The characteristics of uptake and release by the adrenal medulla and synaptic vesicles are consistent with the involvement of this enzyme in the processes (Toll, Gundersen & Howard, 1977). The experimental observations may therefore be explained by the hypothesis set out below. This hypothesis is an attempt to reconcile the effects of the drugs on ATPase activities with their effects on transmitter release and as such it is open to the criticism that the media used for the two types of experiment are different. However, this condition is necessary, because calcium ions, for instance, inhibit $Na^+$, $K^+$-ATPase but are important for transmitter release studies. It should also be noted that the results for membrane $Na^+$, $K^+$-ATPase activities could represent the sum of the activities of more than one such enzyme; we have not attempted to clarify this possibility.

Assume that a membrane $Na^+$, $K^+$-ATPase must be active for evoked (but not basal) transmitter release, and assume that the re-uptake of NA by the nerve terminal depends firstly upon an active $Na^+$, $K^+$-ATPase at the presynaptic membrane and secondly upon an active $Mg^{2+}$-ATPase at the synaptic vesicle. Then, under basal conditions, NA release is increased by ouabain, phenytoin and pemoline (high concentration) since re-uptake is inhibited at the presynaptic membrane. Pemoline at a low concentration stimulates a membrane $Na^+$, $K^+$-ATPase and this could increase re-uptake and diminish the apparent release. For ACh, in the absence of a re-uptake mechanism none of these drugs need have any effect. Where evoked release is concerned, inhibition of a $Na^+$, $K^+$-ATPase by ouabain and phenytoin should prevent or markedly reduce evoked NA release. However the drugs permit the release under evoked conditions to be higher than the basal release (shown as 100% in Figure 8) because some evoked release may continue while re-uptake inhibition occurs. It could be argued that phenytoin, should permit greater release of NA than does ouabain under both basal and evoked conditions because it inhibits vesicular $Mg^{2+}$-ATPase in addition to membrane $Na^+$, $K^+$-ATPase, thereby inhibiting the re-uptake process at two sites. However, this need not be the case, if inhibition of re-uptake at the membrane site is so effective as to reduce the quantitative significance of re-uptake by the vesicles. Pemoline, at a high concentration, gives greater release than ouabain under evoked conditions because of the significantly lower degree of inhibition of $Na^+$, $K^+$-ATPase (maximum inhibition: pemoline, 1 μg/ml, 71%, ouabain, 1 mM, 99%; Gilbert *et al.*, 1978a). The results obtained with ACh are compatible with this hypothesis. The experiments with SC 13504 are also compatible since the drug inhibits vesicular $Mg^{2+}$-ATPase (Gilbert & Wyllie, 1976). Inhibition of the enzyme increased NA release and this is attributed to inhibition of re-uptake of NA (although increased release from vesicles cannot be excluded). When the vesicular $Mg^{2+}$-ATPase inhibitor, SC 13504, and the membrane $Na^+$, $K^+$-ATPase inhibitor, ouabain, were added together the net effect was therefore the same as that of phenytoin alone because phenytoin inhibits both the enzymes inhibited independently by SC 13504 and ouabain. The effects of phenytoin and SC 13504 together or ouabain and phenytoin together were not additive which lends weight to this hypothesis. Experiments are under way to test if these observations are due to the involvement of vesicular $Mg^{2+}$-ATPase in a neurotransmitter pump as suggested by Roufogalis (1973) and whether the involvement is in vesicular release or re-uptake.

The results with the antidepressant drugs are more difficult to interpret. Since all the drugs inhibit $Na^+$, $K^+$-ATPase they should inhibit evoked release of both transmitters and this is the case. We have to propose that the inhibition of basal release by the antidepressants could involve (1) effects of the drugs on the $Na^+$, $K^+$-ATPase of the synaptosome mitochondria, (2) effects on the $Mg^{2+}$-ATPases of the mitochondria and the membrane, (3) inhibition of $Na^+$-ATPase or $Ca^{2+}$-ATPase also present in the synaptosome (none of these enzymes being influenced by ouabain, phenytoin or pemoline) and a fourth possibility is that the antidepressants, which seem much less specific than the other drugs, have effects other

than those on the ATPases reported here. The effects of the antidepressants of (a) increasing NA release under basal conditions and (b) increasing phosphate release when the drugs are present in low concentrations may be due to an effect on an ATPase which we have not yet detected or on some other phosphate releasing process.

We have previously obtained results (Gilbert, Wyllie & Davison, 1975) which are compatible with the hypothesis of Paton *et al.* (1971) that the release of ACh is triggered by inhibition of $Na^+$, $K^+$-ATPase, and we concluded that further work was required. The results of other workers using the isolated spleen of the cat, for instance, are also compatible with the hypothesis (Garcia & Kirpekar, 1975). The experiments of the present work were designed to be more direct tests of the hypothesis and they do not support it. As we have shown in a preliminary communication (Gilbert & Wyllie, 1977) and also demonstrated here, in cerebral cortex slices inhibition of $Na^+$, $K^+$-ATPase did increase ACh release as noted by Vizi (1972). No such effect was detectable in the present work when synaptosomes were used. It seems likely that in slices, inhibition of the enzyme at sites some distance from the nerve terminals leads to depolarization which spreads to the terminals to induce release, whereas direct inhibition of the enzyme in the nerve terminal itself does not trigger release.

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