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The mechanism by which monoamine oxidase inhibitors give rise to a non-calcium-dependent component in the depolarization-induced release of 5-HT from rat brain synaptosomes

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1 The effects of the monoamine oxidase inhibitors pargyline and nialamide on the Ca^{2+}-dependency of [^{3}H]-5-hydroxytryptamine release from superfused rat brain synaptosomes has been studied in order to evaluate the discrepancies that have occasionally been observed in studying transmitter release by *in vivo* and *in vitro* techniques.

2 The application of K^+ pulses of low concentration (12.5–20 mM) caused an essentially Ca^{2+}-dependent release of [^{3}H]-5-HT. However, at K^+ concentrations above 30 mM, a small non-Ca^{2+}-dependent component appeared.

3 At high concentrations of K^+ (30–55 mM), nialamide (18 μM) or pargyline (7 μM) increased the amount of [^{3}H]-5-HT released which could be accounted for by an increase in the non-Ca^{2+}-dependent component of release.

4 The elevation of the non-Ca^{2+}-dependent component of release caused by the monoamine oxidase inhibitors was totally abolished by the inhibitors of the plasma membrane 5-HT carrier, chlomipramine (500 nM), citalopram (50 nM) and fluoxetine (1 μM).

5 The results suggest that the non-Ca^{2+}-dependent component of release seen with high depolarizing concentrations of K^+, particularly in the presence of monoamine oxidase inhibitors, is caused by the efflux of [^{3}H]-5-HT through the plasma membrane carrier which seems to be activated during depolarization.

6 The significance of these findings to the physiological *in vivo* situation, and to the use of *in vitro* preparations in the study of transmitter release is discussed.

Introduction

It has been repeatedly observed that transmitter release *in vivo* in response to depolarization shows an absolute dependency on extracellular Ca^{2+}. In contrast, the release of a number of transmitters studied using *in vitro* preparations has often been shown to be only partially dependent on the availability of extracellular Ca^{2+}. This has been particularly noticeable with γ-aminobutyric acid (GABA) (Cunningham & Neale, 1981; Nelson & Blaustein, 1982; Arias & Tapia, 1986) and dopamine (Liang & Routledge, 1983; Okuma & Osumi, 1986) although the situation with respect to some other transmitters is equivocal (Schoffelmeer & Mulder, 1983; Adam-Vizi & Ligeti, 1984; Arias & Tapia, 1986; Okuma & Osumi, 1986). Whether these findings represent a mode of release that is physiologically relevant, or whether they occur as a consequence of the presence of drugs in the preparation, the mode of stimulation used, or the method of preparation of the tissue is unclear. For example it has been reported that the electrical depolarization of brain slices may provide a more Ca^{2+}-dependent response than that evoked by high concentrations of K^+ (Okuma & Osumi, 1986), and that release produced by veratridine may evoke a greater non-Ca^{2+}-dependent component than K^+ (Cunningham & Neale, 1981; Schoffelmeer & Mulder, 1983; Adam-Vizi & Ligeti, 1984). In addition, the contribution of the non-Ca^{2+}-dependent component to the overall response to depolarization may depend on the treatment of the tissue before the application of the depolarizing stimulus (Nicholls *et al.*, 1987).

Two major interpretations of the non-Ca^{2+}-dependent evoked release of monoamine and amino

acid transmitters have been proposed. Firstly there is evidence that the efflux seen in the absence of extracellular Ca^{2+} may be mediated by the activation of the release mechanism by Ca^{2+} released from mitochondria as a result of the depolarization-induced influx of Na^+. This seems to be particularly relevant to release induced by veratridine (Cunningham & Neale, 1981; Schoffelmeer & Mulder, 1983; Adam-Vizi & Ligeti, 1984). In addition, it has been proposed that the efflux of some transmitters through the relevant plasma membrane carrier occurs under a variety of depolarizing conditions in the absence of extracellular Ca^{2+} (Haycock *et al.*, 1978; Liang & Routledge, 1983; Okuma & Osumi, 1986). It is also noticeable that the inability to demonstrate a clear Ca^{2+}-dependency of evoked monoamine transmitter release seems to occur when monoamine oxidase inhibitors (MAOIs) are used in the preparation (Hery *et al.*, 1983), but not in the absence of drugs (Collard *et al.*, 1981). Furthermore, the size of the non-Ca^{2+}-dependent component of the evoked release of GABA from rat brain slices and frog retina is considerably increased in the presence of an inhibitor of GABA transaminase (Bedwani *et al.*, 1984a,b). It is possible therefore that the non-Ca^{2+}-dependent component of the release of monoamine and some amino acid transmitters can be influenced by events which occur consequent to the inhibition of transmitter metabolism within the nerve terminal. Because there has been a tendency to use inhibitors of transmitter metabolism in a routine manner in studies of transmitter release *in vitro* (e.g. Raiteri *et al.*, 1977; Hollins & Stone, 1980; Fung & Fillenz, 1985; Middlemiss & Spedding, 1985) it seemed pertinent to examine the effect of some of these inhibitors on the characteristics of transmitter release *in vitro*. To this end, the present study has examined the Ca^{2+}-dependency of [^{3}H]-5-HT release from preloaded superfused rat brain synaptosomes as a function of stimulus intensity, and examined the effects of monoamine oxidase inhibitors (MAOIs) on this aspect of release. Experiments were also conducted to explore possible mechanisms responsible for the observed effects of the MAOIs. Some of these results have been published in preliminary form (Collard & Evans, 1987).

Methods

Measurement of 5-HT release from superfused synaptosomes

Male Albino Wistar rats weighing between 200 and 250 g were used in all studies. The methods used to prepare synaptosomes and measure the release of [^{3}H]-5-HT have been described in detail elsewhere (Collard *et al.*, 1981; Suter & Collard, 1983). Synaptosomes were prepared from rat forebrain by the method of Gray & Whittaker (1962) and incubated with 0.1 μM [^{3}H]-5-HT (specific activity 18–19.8 Ci mmol^{-1}) for 10 min at 37°C. Beds of incubated synaptosomes were then set up in perfusion chambers and perfused at 8 ml min^{-1} with oxygenated (95% O_2 + 5% CO_2) Krebs solution as described previously. Serial samples of eluate were collected on ice every 30 s. After 12 min of perfusion, a 38 s pulse of high K^+ Krebs (K^+ replacing Na^+) was applied, after which time the tissue was perfused with the original Krebs solution. At the end of perfusion, the [^{3}H]-5-HT remaining in the tissue was extracted as previously described (Collard *et al.*, 1981), and 2.0 ml portions of each fraction and tissue extract were taken for the separation and measurement of [^{3}H]-5-HT. The efflux of [^{3}H]-5-HT was expressed as a percentage of tissue [^{3}H]-5-HT released per fraction. The release in response to a given degree of depolarisation was calculated as the total [^{3}H]-5-HT released above the baseline during the application of the K^+ pulse (Suter & Collard, 1983).

In studies in which MAOIs were used, the drugs were applied at the incubation stage of the experiment. In studies in which the effects of uptake inhibitors were examined, the drugs were applied at the perfusion stage, so as to allow the prior loading of the tissue with [^{3}H]-5-HT.

Measurement of the inhibition of synaptosomal monoamine oxidase activity by pargyline and nialamide and the selection of doses to use for the release studies

In order to select doses of the MAO inhibitors to use in the release studies, the effectiveness of the drugs in inhibiting the conversion of [^{3}H]-5-HT to [^{3}H]-5-hydroxyindoleacetic acid ([^{3}H]-5-HIAA) in synaptosomes was examined. Synaptosomes were prepared as described above and preincubated for 10 min at 37°C in the presence or absence of nialamide or pargyline (1 μM to 50 μM). [^{3}H]-5-HT (specific activity 16.5 Ci mmol^{-1}) was then added to produce a final concentration of 100 nM, and incubation continued for a further 10–30 min. Since 5-HIAA is rapidly cleared from the nerve ending (Collard *et al.*, 1981), the activity of MAO was determined by measuring the rate of appearance of [^{3}H]-5-HIAA in the extracellular phase. This was accomplished by centrifuging the incubated synaptosomes at 10,000 *g* for 10 min and separating and measuring the amount of [^{3}H]-5-HT and [^{3}H]-5-HIAA present in aliquots of supernatant by ion exchange chromatography as described previously (Collard *et al.*, 1981). Dose-response curves of the percentage inhibi-

tion of the conversion of [^{3}H]-5-HT to [^{3}H]-5-HIAA by pargyline and nialamide were plotted, and doses of the two drugs that gave a similar and significant degree of inhibition were selected from the curves. These doses were, for nialamide 18 μM, which gave a percentage inhibition of 82.0 $\pm$ 1.33, and for pargyline 7 μM, which gave a percentage inhibition of 81.1 $\pm$ 0.233 (n = 3). These doses were used in the relevant release experiments.

The measurement of the inhibition of [^{3}H]-5-HT uptake by citalopram, chlomipramine, and fluoxetine, and the selection of doses to use in the release studies

The uptake of [^{3}H]-5-HT was measured as described previously (Wilkinson & Collard, 1984). Uptake was analysed by a curve-fitting procedure which confirmed that the uptake could be accounted for by a single carrier-mediated process that obeyed Michaelis-Menton kinetics (Munson & Rodbard, 1980). The curve fitting procedure also provided values for the kinetic constants. These were a K_m of 74.7 nM and a V_{max} of 2.31 pmol mg^{-1} protein 10 s^{-1}. In the assessment of the inhibitory potency of the uptake blockers, synaptosomes were preincubated with or without one of the drugs for 5 min. [^{3}H]-5-HT was then added to the suspension to provide a final concentration of 50 nM. Incubation was continued for a further 10 s before being processed as described previously (Wilkinson & Collard, 1984). The effect of a range of doses of each drug was studied. These were for citalopram 1–100 nM, for chlomipramine 10–1000 nM and for fluoxetine 50 nM–5 μM. From the resulting dose-response curves, doses of the inhibitors were selected that gave comparable degrees of inhibition of uptake (approximately 60%). These were for citalopram, 50 nM (64.5 $\pm$ 2.0% inhibition, n = 5), chlomipramine 500 nM (60.5 $\pm$ 3.0% inhibition, n = 5) and fluoxetine 1 μM (57.3 $\pm$ 1.6% inhibition, n = 5). The inhibitors were used in these concentrations in the later studies which examined the involvement of the plasma membrane 5-HT carrier in the efflux of 5-HT in the presence and absence of MAO inhibitors.

Drugs

Pargyline and nialamide were obtained from Sigma. The inhibitors of the plasma membrane 5-HT carrier were obtained as gifts from the following pharmaceutical companies. Ciba-Geigy (chlomipramine), Lundbeck and Co. Copenhagen (citalopram), Eli Lilly and Co. Indianapolis (fluoxetine). [^{3}H]-5-HT creatinine sulphate (generally labelled) was obtained from Amersham International.

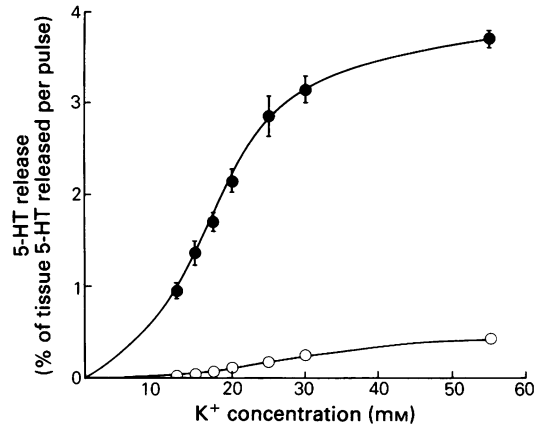

Figure 1 The release of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) evoked by the application of a 38 s pulse of K^+ at concentrations ranging from 12.5 mM to 55 mM in the presence (●) or absence (○) of extracellular Ca^{2+}. Release is expressed as the % of tissue [^{3}H]-5-HT released per pulse (mean with s.e.mean shown by vertical bars, n = 5). Where error bars are not shown they fall within the symbol.

Results

The calcium-dependency of [^{3}H]-5-HT release as a function of K^+ concentration

The release of [^{3}H]-5-HT in response to the application of a 38 s pulse of K^+ at concentrations from 12.5–55 mM in the presence (2.5 mM) or absence of extracellular Ca^{2+} is given in Figure 1. It can be seen that a small non-Ca^{2+}-dependent component of the evoked release does appear but only at the higher levels of stimulation used.

The effect of the MAO inhibitors pargyline and nialamide on the calcium-dependency of [^{3}H]-5-HT release

The effect of nialamide (18 μM) and pargyline (7 μM) on the release of [^{3}H]-5-HT in response to the application of 20 mM, 30 mM and 55 mM K^+ in the presence or absence of Ca^{2+} is shown in Figure 2. The release of [^{3}H]-5-HT in response to depolarization both in the presence and absence of extracellular Ca^{2+} was larger when the MAO inhibitors were present in the perfusion fluid. It can also be seen from Figure 2 that this effect was greater at the highest intensity of stimulation used (55 mM K^+). The overall increase in release seen in the presence of the MAO inhibitors was not significantly different

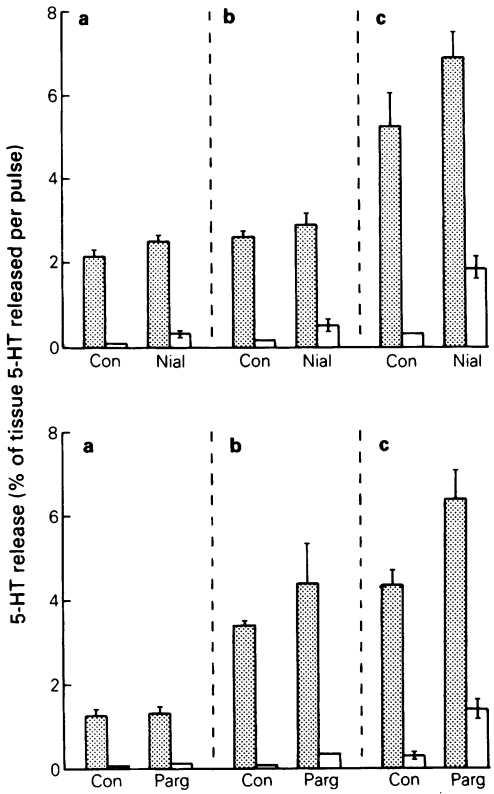

Figure 2 The release of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) caused by the application of a 38 s pulse of 20 mM K^+ (a), 30 mM K^+ (b) and 55 mM K^+ (c), in the presence (shaded columns) or absence (open columns) of extracellular Ca^{2+}. The release in the presence (Nial) or absence (Con) of nialamide is shown in the upper histogram. The release in the presence (Parg) or absence (Con) of pargyline is shown in the lower histogram. The results are expressed as the % of tissue [^{3}H]-5-HT released per pulse (mean with s.e.mean shown by vertical bars, $n = 4$ or 5).

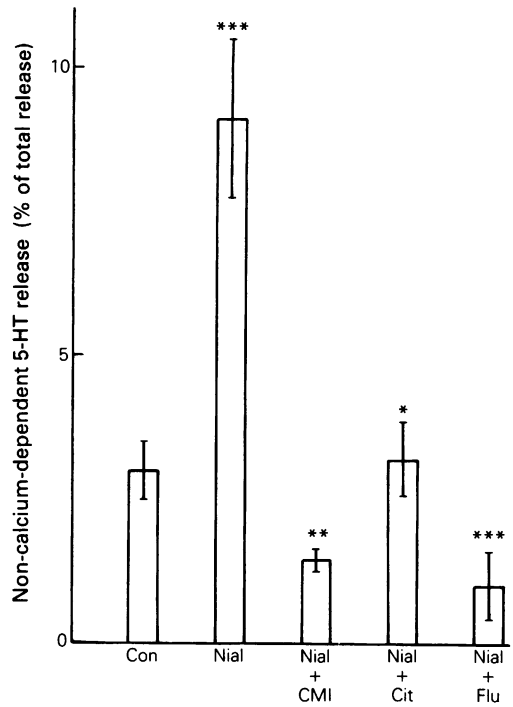

Figure 3 The non-Ca^{2+}-dependent release of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) in response to a 38 s pulse of 55 mM K^+ in drug-free (Con) conditions and in the presence of nialamide alone (Nial) or nialamide plus chlomipramine (Nial + CMI), nialamide plus citalopram (Nial + Cit) or nialamide plus fluoxetine (Nial + Flu). The non-Ca^{2+}-dependent component of release is expressed as the mean percentage of the total release ± s.e.mean (vertical bars) ($n = 12$ for the control and nialamide alone, and $n = 4$ or 5 for the nialamide + uptake inhibitors). Significant differences between the control and nialamide-treated synaptosomes is given as $^{***}P < 0.001$. Significant differences between the nialamide-treatment alone or in the presence of inhibitors of uptake is given as $^{*}P < 0.02$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Student's t test.

from that seen just in the non-Ca^{2+}-dependent component of the response. In contrast to the effects seen on the K^+-evoked release of [^{3}H]-5-HT, neither drug had any significant effect on the basal efflux of the transmitter.

The effect of inhibitors of the plasma membrane 5-HT carrier on the nialamide-induced elevation of the non-Ca^{2+}-dependent component of [^{3}H]-5-HT release in response to a pulse of 50 mM K^+

The test situation used in this study was the release of [^{3}H]-5-HT in response to a pulse of 55 mM K^+. As demonstrated in the previous experiment, this provided a large non-Ca^{2+}-dependent response in the presence of 18 μM nialamide. The effects of the inhibitors of the plasma membrane 5-HT carrier on the ability of nialamide to increase the relative size of the non-Ca^{2+}-dependent component of the evoked release is shown in Figure 3. In the presence of nialamide, the non-Ca^{2+}-dependent component increased from 3.0% of the total release to 9.1% This was a somewhat smaller increase than that seen in the previous study but it was nevertheless a sig-

nificant increase ($P < 0.001$). This effect of nialamide was totally abolished by chlomipramine (500 nM), citalopram (50 nM) and fluoxetine (1 μM). The ability of citalopram to reduce the effect of nialamide was however significantly lower than that seen with the other two inhibitors ($P < 0.05$, Student's t test).

In addition to inhibiting the effect of nialamide, the inhibitors also reduced the relative size of the smaller non-Ca^{2+}-dependent component of the response to depolarisation in the absence of the MAO inhibitor. In the presence of chlomipramine, fluoxetine or citalopram, the non-Ca^{2+}-dependent component amounted to $0.259\% \pm 0.022$, $0.228\% \pm 0.045$ and $2.154\% \pm 0.446$ respectively of the response in the presence of Ca^{2+} ($n = 4$). As with the effects seen in the presence of nialamide, the largest reductions were seen with fluoxetine and chlomipramine, and these differed significantly from that of citalopram ($P < 0.05$, Student's t test).

Discussion

The application of K^+ pulses of low concentration (12.5–20 mM) caused an essentially Ca^{2+}-dependent release of [^{3}H]-5-HT. This resembles what might be expected to occur in the physiological *in vivo* situation. However, at K^+ concentrations above 30 mM, a small non-Ca^{2+}-dependent component appeared. Nialamide (18 μM) and pargyline (7 μM) increased both the total amount of [^{3}H]-5-HT released in response to depolarization and the non-Ca^{2+}-dependent component of the release. The data further implied that the overall increase in release in the presence of the MAO inhibitors could be accounted for to a large extent by the increase seen in the non-Ca^{2+}-dependent component. The most likely explanations for these observations are that either the route through which 5-HT leaves the nerve ending in the Ca^{2+}-free environment is activated by the MAO inhibitors or that more 5-HT is made available for the route. Since the rate of metabolism of [^{3}H]-5-HT is quite high in synaptosomes maintained under the conditions used in these experiments (Collard *et al.*, 1981) the inhibition of 5-HT metabolism would provide more 5-HT for an efflux pathway for which there is little 5-HT available in the drug-free situation. The ability of a series of inhibitors of the plasma membrane 5-HT carrier to abolish the effect of the MAO inhibitors suggests that 5-HT is using the carrier as a route of release under these experimental conditions. In a similar study of the release of endogenous dopamine from brain slices, Okuma & Osumi (1986) showed that the non-Ca^{2+}-dependent component of release seen in the presence of pargyline could also be inhibited by the dopamine transport inhibitor nomifensine. In the present study it is unlikely that the effect of the inhibitors of the carrier was mediated by blocking the entry of the MAO inhibitors into the nerve endings because the synaptosomes were exposed to the MAO inhibitors for 30 min prior to being set up in the perfusion chambers and perfused with fluid containing the uptake blockers. Furthermore, the finding that the uptake inhibitors also reduced the size of the non-Ca^{2+}-dependent efflux of [^{3}H]-5-HT in control synaptosomes would further support the view that the carrier is available in drug-free conditions but that the amount of 5-HT available to it is limited. Thus the most likely interpretation of these observations is that the inhibition of 5-HT metabolism leads to an increase in the amount of 5-HT which is able to leave the nerve ending by the plasma membrane 5-HT carrier.

The finding that the MAO inhibitors had little effect on the basal efflux of [^{3}H]-5-HT would imply that the activity of the carrier is greater during periods of depolarization than in the unstimulated state. The activation of plasma membrane carriers for some transmitters by K^+ or veratridine depolarization has been observed on many previous occasions (Haycock *et al.*, 1978; Nelson & Blaustein, 1982; Liang & Routledge, 1983; Oksuma & Osumi, 1986) although not all transmitters show this phenomenon, and the carrier mediated release of dopamine from brain slices did not occur following electrical depolarization (Oksuma & Osumi, 1986). In all the studies showing clear evidence of carrier-mediated release alluded to previously, the amount of transmitter available to the carrier was increased by using inhibitors of transmitter metabolism. This carrier-mediated efflux of transmitter has been interpreted as an efflux pathway which contributed to the physiological release process (Nelson & Blaustein, 1982). An alternative interpretation is that the efflux of transmitter through the activated carrier may be an artifact of the way in which the experiment was conducted. In a previous study by Okuma & Osaki (1986), carrier-mediated efflux of endogenous dopamine from brain slices occurred following K^+-depolarization, but not in response to electrical depolarization. In the present study, the contribution to the overall release of transmitter made by the carrier-mediated process was only significant at high depolarizing concentrations of K^+ and in the presence of inhibitors of MAO. Thus we propose that in the physiological situation where no drugs are present, and the stimulus is in the form of action potentials of short duration, it is likely that little transmitter leaves the nerve ending via the plasma membrane carrier. On this point, it is noticeable that one type of cell which does show good evidence of a

physiological role for carrier-mediated efflux is the horizontal cell of the fish retina (Schwartz, 1987). These cells do not propagate action potentials and respond physiologically with more long-term changes in membrane potential than classical nerve cells. It may well be that carrier-mediated efflux of transmitters becomes a component of release in response to more long-term depolarizing influences either physiologically as in horizontal cells, or artifactually as in *in vitro* conditions in which brain tissue is exposed to chemical depolarizing stimuli for many seconds.

The results of this study do show however, that it is still possible to produce a pattern of release *in vitro* which resembles the physiological state by ensuring that only mild to moderate intensities of K^+ depolarization are used, and that the use of MAO inhibitors (and possibly other inhibitors of transmitter metabolism) is avoided. It might be considered that a similar experimental protocol could be achieved by using inhibitors of both MAO and uptake together. However, this would lead to an unphysiological intracellular distribution of 5-HT which could influence intraneuronal 5-HT homeostasis. Furthermore, the interpretation of the effects of other drugs and endogenous modulators on 5-HT release would be very difficult in a preparation already compromised by the presence of drugs. It is thus much simpler to use the more physiological drug-free experimental situation outlined above which overcomes all of these problems and allows a more direct examination of the effects of experimental manipulations on 5-HT release. A similar word of caution concerning the use of inhibitors of transmitter metabolism has also been expressed by Okuma & Osumi (1986) following their studies on dopamine release.

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