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On the mechanism by which extracellular sodium depletion causes 5-hydroxytryptamine release from rat brain synaptosomes

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The release of ^{3}H-labelled 5-hydroxytryptamine (5-HT) from preloaded and superfused rat forebrain synaptosomes in response to extracellular Na^+ depletion was studied. In the absence of monoamine oxidase inhibitors, the release of [^{3}H]-5-HT caused by Na^+ depletion was not affected by immobilisers of the plasma membrane 5-HT carrier. The release of [^{3}H]-5-HT in response to Na^+ depletion was also either independent of, or inversely related to the concentration of extracellular Ca^{2+} depending on the degree to which extracellular Na^+ was reduced. The efflux of $^{45}Ca^{2+}$ from prelabelled synaptosomes was decreased by Na^+ reduction but the amplitude of the changes in $^{45}Ca^{2+}$ efflux did not totally correlate with the changes in [^{3}H]-5-HT efflux under the same experimental conditions. These results suggest that the release of [^{3}H]-5-HT caused by Na^+ depletion in drug-free synaptosomes is not mediated by 5-HT efflux through the plasma membrane carrier, nor to changes in cytosolic Ca^{2+} consequent to changes in Ca^{2+} fluxes across the plasma membrane. The results have been tentatively explained as an elevation of spontaneous 5-HT efflux caused by an increase in membrane fluidity mediated by the ionic manipulations used to produce the Na^+-depleted media.

Introduction

Decreasing extracellular Na^+ concentration has been shown to cause the release of 5-hydroxytryptamine (5-HT) and other monoamine transmitters from nerve-ending preparations in vitro [1–4]. This has been interpreted as being due to the efflux of cytoplasmic 5-HT via the plasma membrane 5-HT carrier caused by a change in the trans-membrane Na^+ electrochemical gradient [1–4]. In all the above studies, the amount of intracellular monoamine which could gain access to the carrier molecule was increased by the use of monoamine oxidase inhibitors with or without reserpine to release the monoamine from storage. Recent work in our laboratory has shown that in the absence of such drugs the amount of 5-HT which can use the carrier to move out of the cell is very limited [5]. Consequently, under these experimental conditions, the release of transmitter in response to a decrease in extracellular Na^+ is likely to be mediated independently of the plasma membrane carrier. Because changes in the Na^+ gradient can also influence Ca^{2+} flux across the nerve terminal membrane [6–8], it is possible that the effect of extracellular Na^+ depletion on 5-HT release may be Ca^{2+} mediated. There is strong evidence that one of the processes by which intracellular Ca^{2+} may be maintained at a low resting level is a plasma membrane Na^+-Ca^{2+} exchange process by which Na^+ entering the nerve down the electrochemical gradient drives Ca^{2+} out of the cell [7,8]. Dissipation of the Na^+ gradient will decrease Ca^{2+} efflux [9] and increase Ca^{2+} uptake into the cell [6,10]. A consequence of this might be an increase in intracellular Ca^{2+} sufficient to trigger some transmitter release [7]. Were this to be so, release elicited in such a way might be a useful technique to use in determining the site of action of modulators of transmitter release.

This study was therefore undertaken in order to determine the relative contributions made by carrier-mediated and Ca^{2+}-mediated processes in the release of 5-HT from rat forebrain synaptosomes caused by decreasing extracellular Na^+.

Preliminary accounts of some of this work have been published previously [11,12].

Abbreviations: 5-HT, 5-hydroxytryptamine; PCA, perchloric acid.

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Methods

Preparation of synaptosomes

Male Albino Wistar rats weighing between 200 g and 250 g were used in all studies. Synaptosomes were prepared from whole forebrain by a modification of the method of Gray and Whittaker [13] as previously described [14]. The final synaptosomal pellet (P_2-B) was resuspended in Krebs solution of varying volume and composition depending on the individual experiment (see below).

Measurement of 5-HT efflux

The methods used to study the release of accumulated [^{3}H]-5-HT were essentially as described previously [14,15]. The synaptosomal pellet was resuspended in 20 ml of Krebs solution of the following composition (mM): NaCl, 124, KCl, 5.0; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 0.75; glucose, 10; and $NaHCO_3$, 26.0. A 10 ml portion was then incubated with 100 nM [^{3}H]-5-HT (spec. act. 12–14 Ci/mmol) for 10 min at 37°C. After incubation, 4.5-ml portions of the synaptosomal suspension were removed for the preparation of synaptosome beds and placed in perfusion chambers as previously described [14]. The beds of synaptosomes were perfused at 8.0 ml/min with oxygenated (95% O_2 + 5% CO_2) Krebs of varying composition depending on the experiment. Previous studies have shown that at this rate of perfusion, the recapture of released [^{3}H]-5-HT does not occur [14]. Consequently any changes in efflux observed in response to the manipulations conducted in the course of these experiments may be confidently assumed to be due to changes in release, and not a consequence of changes in uptake activity.

The composition of the major solutions used is given in Table I. In most experiments, the synaptosomes were initially perfused with the standard Krebs solution. Thereafter, pulse applications of low-Na^+ Krebs were administered as described in subsequent sections. The involvement of the other solutions given in Table I will be mentioned at the appropriate point in later sections. All solutions described in Table I were isosmotic with the standard Krebs solution.

Throughout the period of perfusion, serial samples of eluant were collected on ice every 30 s. At the end of the period of perfusion, the synaptosome bed was homogenised in 5.0 ml of 0.4 M perchloric acid and centrifuged at $1000 \times g$ for 10 min. A 2.0 ml portion of the supernatant was removed and adjusted to pH 7.5 with NaOH. The [^{3}H]-5-HT present in the tissue extract and in 2.0 ml samples of each fraction of collected perfusate was separated using a modification of the method of Smith et al. [16], as described previously [14]. Separated [^{3}H]-5-HT was mesured by liquid scintillation counting. The efflux of [^{3}H]-5-HT was expresed as a percentage of the tissue [^{3}H]-5-HT released per fraction. The amount of [^{3}H]-5-HT released in response to a pulse of low-Na^+ Krebs solution was calculated as the total amount of [^{3}H]-5-HT released above the baseline during the application of the pulse.

This method of assessing 5-HT release assumes that the accumulated [^{3}H]-5-HT labels the physiologically relevant 5-HT pools and that the measured release of [^{3}H]-5-HT is a faithful monitor of the release of endogenous transmitter. While this is the case under a number of experimental conditions [5,14], there are others in which this assumption cannot be made [17]. The present experiments have been designed to eliminate those conditions in which a disparity between the release of tritiated and endogenous transmitter is likely to occur [17]. This provides a high level of confidence in the data derived from these experiments. It should be noted

TABLE I

The composition in mM of the major perfusion fluids used

Component	Standard Krebs	Na^+-fre Krebs (standard)	30 mM Na^+ Krebs	Na^+-free choline Krebs	Tris-based Krebs with Na^+	Low-Cl^- Krebs
NaCl	121	–	30	–	115	–
KCl	4.85	4.85	4.85	4.85	4.85	4.85
KH_2PO_4	1.15	1.15	1.15	1.15	1.15	1.15
$MgSO_4$	1.15	1.15	1.15	1.15	1.15	1.15
$CaCl_2$	1.00	1.00	1.00	1.00	1.00	1.00
Glucose	11.1	11.1	11.1	11.1	11.1	11.1
$NaHCO_3$	25.0	–	–	–	–	–
Sucrose	–	188	141.6	–	–	–
Tris base	–	11.0	11.0	11.0	11.0	11.0
Tris-HCl	–	26.0	26.0	26.0	26.0	26.0
Sodium isethionate	–	–	–	–	–	121
Choline chloride	–	–	–	121	–	–
Ionic strength	0.162	0.035	0.065	0.152	0.150	0.151

however, that it is never possible to be absolutely certain that all the changes in [^{3}H]-5-HT release seen in experiments of this type parallels that which would be expected of the endogenous transmitter.

Measurement of $^{45}Ca^{2+}$ efflux

The synaptosomal pellet was resuspended in 20 ml of standard Krebs solution (Table I). 9.9-ml portions were preincubated for 10 min at 37°C. 100 µl of standard Krebs solution containing $^{45}Ca^{2+}$ at a specific activity of 172.4 µCi/µmol were then added to the suspension and incubation continued for another 10 min at 37°C. After this time, 4.5-ml portions of incubated synaptosomes were removed for the preparation of synaptosome beds as described above. The synaptosome beds were perfused at 8.0 ml/min with standard Krebs solution or Ca^{2+}-free Krebs solution (Ca^{2+} replaced isosmotically with Na^+). The solutions were constantly gassed with 95% O_2 and 5% CO_2. At times given in the individual experiments (see Results), the synaptosomes received pulse applications of low-Na^+ or Na^+-free Krebs solutions. Serial 30-s fractions of eluate were collected. At the end of the experiment, the tissue was homogenised in 0.4 M PCA. The homogenate was centrifuged at 1000 × *g* for 10 min. 1.0-ml portions of the collected perfusate and tissue extract were then removed and the amount of $^{45}Ca^{2+}$ present determined by liquid scintillation counting. Efflux was usually expressed in the same way as 5-HT efflux, i.e. given as a percentage of tissue $^{45}Ca^{2+}$ release per fraction.

Measurement of Na^+ concentration in the perfusate

The Na^+ concentration of the samples of collected perfusate during the various experimental manipulations was measured by atomic emission spectrophotometry using an EEL Flame Photometer.

Drugs and isotopes

The following inhibitors of the plasma membrane 5-HT carrier were obtained as gifts from the following pharmaceutical companies: chlorimipramine (Ciba-Geigy), citalopram (Lundbeck & Co., Copenhagen), fluoxetine (Eli Lilley & Co., Indianapolis). [^{3}H]-5-HT creatinine sulphate (generally labelled) and $^{45}Ca^{2+}$ were obtained from Amersham International.

Results

Verification of the methodology used to study the effect of Na^+ removal on [^{3}H]-5-HT release

A number of preliminary studies were necessary to confirm that the experimental procedure adopted for the major study was actually studying the effect of Na^+ removal, and not a consequence of the other manipulations necessary to produce a low Na^+ environment of the correct osmolarity and buffering power. The standard low-Na^+ medium replaced NaCl isosmotically with sucrose, and $NaHCO_3$ with the organic Tris buffer system (see Table I). The first experiment compared the effect of the application of a 2 min pulse of Na^+-free

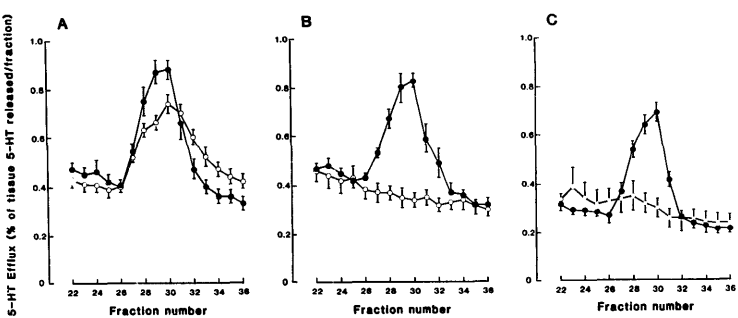

Fig. 1. The efflux of [^{3}H]-5-HT from superfused synaptosomes caused by the application of 2-min pulses of Krebs solutions of various composition. Synaptosomes prelabelled with [^{3}H]-5-HT were perfused at 8 ml/min and the [^{3}H]-5-HT present in 30-s fractions of collected perfusate measured. In (A), (B), and (C), the control response to the application (at fraction 25) of a 2 min pulse of Na^+-free Krebs in which Na^+ had been replaced by sucrose and Tris is shown in closed circles. In (A) the response is compared to that produced by a Na^+-free solution in which Na^+ had been replaced by choline chloride and Tris (○). In (B) the response is compared to that produced by a solution containing Na^+ and Tris (○). In (C) the response is compared to that produced by a 2 min pulse of Krebs solution containing sodium isethionate in place of NaCl (low-Cl^- solution) (○). Efflux is expressed as the percentage of tissue [^{3}H]-5-HT released per fraction. Results are given as the mean ± S.E. of nine observations (A), or five observations (B and C).

Krebs of the above composition with that caused by the application of a pulse of Na^+-free Krebs in which NaCl had been replaced by choline chloride. The pulse of Na^+-free solution was applied at fraction 25. The results of the study is shown in Fig. 1A. It can be seen that although there were slight differences in the form of the response, the application of either of the Na^+-free solutions caused a quantitatively similar release of [^{3}H]-5-HT (see also Table II). Both of the above solutions used a Tris-based buffering system. The effect of the presence of Tris was assessed by comparing the response to a 2 min pulse of Na^+-free Krebs with the response to a pulse of Krebs solution containing both Na^+ and Tris. It was not possible to completely replace Na^+ because of the absence of $NaHCO_3$, consequently a solution containing 115 mM Na^+ (see Table I) was used. The result of the study is shown in Fig. 1B. It can be seen that in contrast to the effect of the Na^+-free pulse, the application of a pulse of Krebs solution containing Na^+ and Tris did not cause the release of [^{3}H]-5-HT. Finally, it was important to determine whether the response to the Na^+-free (sucrose-based) solution was due to the absence of Na^+ or the reduction in Cl^-. The finding that replacement of NaCl with choline chloride still produced a comparable release would argue against the effects being mediated by a decrease in extracellular Cl^-. However, it has been reported [18] that replacing Cl^- with impermeant anions caused the release of transmitters from synaptosomes and brain slices, and that this effect was probably related to the depolarising effect of reducing extracellular Cl^-. The effect of reducing Cl^- but maintaining Na^+ was examined by comparing the effects of a pulse of Na^+-free Krebs with that of a pulse of Krebs solution in which NaCl had been replaced isotonically with sodium isethionate. Isethionate has been routinely used as an impermeant ion for Cl^- replacement studies on neural tissue [19]. The result of this study is shown in Fig. 1C. It can be seen that while the application of the Na^+-free caused a significant release of [^{3}H]-5-HT, the application of a pulse of Na^+-rich Krebs containing isethionate had little effect. This suggests that the replacement of Cl^- with the impermeant anion isethionate did not depolarise the synaptosomes sufficiently to cause a measurable release of 5-HT under the conditions used in this experiment.

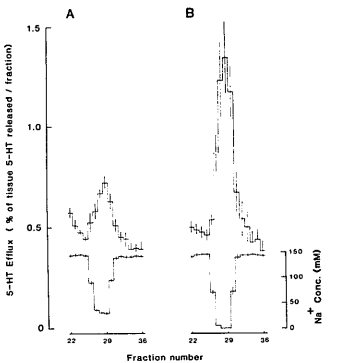

Fig. 2. The release of [^{3}H]-5-HT in response to the pulse application of Na^+-depleted medium as a function of the change in the Na^+ concentration of the perfusion fluid. Synaptosomes prelabelled with [^{3}H]-5-HT were perfused at 8 ml/min and the [^{3}H]-5-HT and Na^+ present in 30-s fractions of collected perfusate measured. The upper trace shows the release of [^{3}H]-5-HT in response to the application of a 2 min pulse of Krebs solution containing 30 mM Na^+ (A), or Na^+-free Krebs (B). In both cases, Na^+ was replaced by sucrose and Tris. The lower trace shows the Na^+ concentration of the collected perfusate under the same experimental conditions. The results are given as the mean ± S.E. ($n = 4$).

The results of these preliminary studies determined that the effect of Na^+ removal on 5-HT release using sucrose-based Krebs solution was due to the absence of Na^+ rather than to the absence of Cl^-, the presence of Tris or the presence of sucrose. Because choline is neurally active, the Na^+-free solution used for most of the routine studies was one in which Na^+ was replaced isosmotically with sucrose and Tris (Table I).

The release of [^{3}H]5-HT in response to the application of pulses of 30 mM Na^+ Krebs and Na^+-free Krebs: correlation with Na^+ concentration

The result of this study is shown in Fig. 2. The amount of [^{3}H]-5-HT released in response to a pulse of Krebs solution containing 30 mM Na^+ was significantly less than that caused by a pulse of Na^+-free Krebs solution under similar experimental conditions. Furthermore, the amount of [^{3}H]-5-HT release in response to the application of the low-Na^+ solutions correlated well with the magnitude of the decrease in the extracellular Na^+ level caused by the manipulations.

The release of [^{3}H]-5-HT in response to the application of pulses of low-Na^+ Krebs solutions at varying levels of extracellular Ca^{2+}

The purpose of this study was to provide an indication of the involvement of Ca^{2+} entry into the nerve ending in the responses to extracellular Na^+ depletion. The effect of pulses of 30 mM Na^+ and Na^+-free Krebs solution were examined at extracellular Ca^{2+} concentrations ranging from 0 to 2.5 mM. The results of the study are shown in Fig. 3. It can be seen that the release of [^{3}H]-5-HT in response to 30 mM Na^+ was similar at all concentrations of extracellular Ca^{2+} studied. In con-

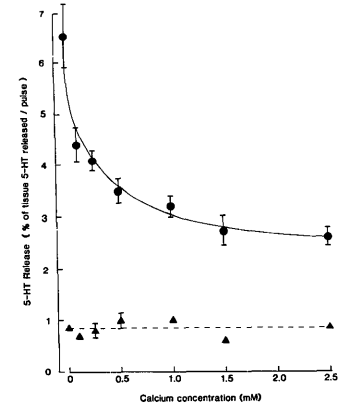

Fig. 3. The release of [^{3}H]-5-HT in response to the application of a 2 min pulse of Na^+-free or 30 mM Na^+ Krebs solution as a function of extracellular Ca^{2+} concentration. The release of [^{3}H]-5-HT is expressed as the total amount of tissue [^{3}H]-5-HT released per pulse and given as the mean ± S.E. of four or five observations. The release in response to the application of 30 mM Na^+ Krebs is shown with closed triangles, and that in response to the Na^+-free pulse with closed circles.

trast, the amount of [^{3}H]-5-HT released in response to the application of a Na^+-free pulse appeared to be inversely related to the concentration of extracellular Ca^{2+}. A later study compared the release of [^{3}H]-5-HT in response to 2-min pulses of sucrose substituted or choline substituted Na^+-free and 30 mM Na^+ Krebs with or without Ca^{2+} (1.0 mM). The results are shown in Table II. In both cases, the release of [^{3}H]-5-HT was significantly greater in Ca^{2+}-free fluid than in fluid containing 1.0 mM Ca^{2+}.

TABLE II

The effect of extracellular Ca^{2+} on the release of [^{3}H]-5-HT caused by choline or sucrose replacement of extracellular Na^+

The release of [^{3}H]-5-HT is expressed as the total amount of tissue [^{3}H]-5-HT released per pulse, and given as the mean ± S.E. of five observations. Significant differences between the release in Ca^{2+}-free and 1.0 mM Krebs solution is given by * $P < 0.01$, ** $P < 0.001$ (Student's t-test).

Treatment	Ca^{2+} free	1.0 mM Ca^{2+}
Sucrose substitution	2.690 ± 0.209	1.762 ± 0.123 *
Choline substitution	2.844 ± 0.088	1.693 ± 0.080 **

The effect of inhibition of the plasma membrane 5-HT carrier on the release of [^{3}H]-5-HT in response to pulses of Na^+-free Krebs solutions

In order to determine the involvement of the plasma membrane 5-HT carrier in the efflux of [^{3}H]-5-HT caused by extracellular Na^+ depletion this study examined the effect of a range of inhibitors of the 5-HT carrier on the release of [^{3}H]-5-HT. The drugs used ranged from a relatively specific and very potent inhibitor citalopram to a very specific but not very potent inhibitor fluoxetine [20]. Previous studies had determined doses of the drugs which provided equivalent degrees of inhibition of [^{3}H]-5-HT influx by the carrier [5]. These were for citalopram 50 nM, chlomipramine 500 nM, and fluoxetine 1 μM. Since carrier-mediated transport is a two-way process with the binding of substrate (or inhibitor) occurring at both the inner and outer surface of the membrane during any complete transport cycle, it does not matter at which site the inhibitor binds to the carrier. Furthermore, the outward transport of substrate will be expected to be inhibited to a similar degree to that of the inward transport observed in the uptake studies [5], and the inhibitors could consequently be used to test the involvement of the carrier in the outward transport of [^{3}H]-5-HT in response to extracellular Na^+ depletion. In each experiment, the response in control beds of synaptosomes was compared to those in which the perfusion fluid contained one of the drugs in the concentration given above. The results of this study are given in Table III. It can be seen that under these experimental conditions, the release of [^{3}H]-5-HT in response to the Na^+-free pulse was little affected by the presence of the uptake inhibitors. In contrast to these findings, when the effect of fluoxetine was examined in the presence of the monoamine oxidase inhibitor nialamide, the release of

TABLE III

The effect of immobilisation of the plasma membrane 5-HT carrier on the release of [^{3}H]-5-HT in response to Na^+ depletion

The release of [^{3}H]-5-HT is expressed as the total amount of tissue [^{3}H]-5-HT released per pulse and given as the mean ± S.E. The number of observations is given in parentheses. Significant difference between the release of [^{3}H]-5-HT in the presence of nialamide alone and that in the presence of nialamide and fluoxetine is given by * $P < 0.05$ (Student's t-test). There were no significant differences between the control response and the release in the presence of the uptake inhibitors alone.

Treatment	[^{3}H]-5-HT release
Control	1.218 ± 0.078 (20)
Citalopram (50 nM)	1.127 ± 0.192 (5)
Chlorimipramine (500 nM)	1.256 ± 0.198 (4)
Fluoxetine (1 μM)	1.183 ± 0.077 (5)
Nialamide (18 μM)	1.296 ± 0.141 (5)
Nialamide (18 μM) + fluoxetine (1 μM)	0.742 ± 0.156 (5) *

[^{3}H]-5-HT evoked by a pulse of Na^+-free Krebs was clearly reduced. Interestingly, the overall release of [^{3}H]-5-HT in response to Na^+ depletion in the presence of nialamide was only slightly increased when compared with the control, but the contribution made by the carrier was clearly increased.

The effect of the application of Na^+-free and 30 mM Na^+ pulses on the efflux of $^{45}Ca^{2+}$ from preloaded superfused synaptosomes

This experiment was designed to determine whether there was any correlation between [^{3}H]-5-HT release and $^{45}Ca^{2+}$ efflux caused by total and partial (30 mM) Na^+ depletion at varying levels of extracellular Ca^{2+}. Consequently, the effect of the application of Na^+-free or 30 mM Na^+ pulses on the efflux of $^{45}Ca^{2+}$ from preloaded synaptosomes was studied in Ca^{2+}-free Krebs solution, and in Krebs solution containing 1.0 mM Ca^{2+} (standard Krebs, Table I). Due to the active nature of $^{45}Ca^{2+}$ efflux, the tissue levels of $^{45}Ca^{2+}$ declined very rapidly. Because of this, it was difficult to accurately study the effects of the application of pulses applied at fraction 25. Consequently, in this study, a 2 min pulse of the Na^+-depleted Krebs solution was applied at fraction 15. This permitted enough time for the basal efflux of $^{45}Ca^{2+}$ to reach a relatively steady level before the application of the pulse. The results of this study (Fig. 4) showed that both the resting efflux of $^{45}Ca^{2+}$ and the decrease in efflux caused by Na^+ reduction was greater in Ca^{2+}-free fluid than in fluid containing 1.0 mM Ca^{2+}. Following the replacement of Na^+ there was a rebound increase in efflux. This difference in $^{45}Ca^{2+}$ efflux seen between Ca^{2+}-free and 1.0 mM Ca^{2+} Krebs was similar whether the Na^+ was reduced to zero or to 30 mM. With respect to the release of [^{3}H]-5-HT caused by total Na^+ depletion, these results show a good correlation between changes in $^{45}Ca^{2+}$ efflux and transmitter release. However, the release of [^{3}H]-5-HT caused by reducing extracellular Na^+ to 30 mM did not vary with extracellular Ca^{2+} despite marked changes in $^{45}Ca^{2+}$ efflux.

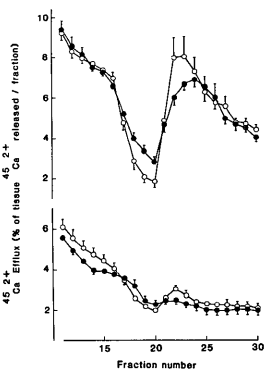

Fig. 4. Changes in the efflux of $^{45}Ca^{2+}$ from preloaded superfused synaptosomes in response to the application of a 2 min pulse of Na^+-free or 30 mM Na^+ Krebs fluid in the presence or absence of extracellular Ca^{2+}. Synaptosomes prelabelled with $^{45}Ca^{2+}$ were perfused at 8 ml/ml and the $^{45}Ca^{2+}$ present in 30-s fractions of collected perfusate measured. A 2 min pulse of Na^+-depleted Krebs fluid was applied at fraction 15. The efflux of $^{45}Ca^{2+}$ in synaptosomes perfused with Ca^{2+}-free Krebs is shown in the upper graph, while that in synaptosomes perfused with Krebs containing 1.0 mM Ca^{2+} is shown in the lower graph. In both graphs, the efflux in synaptosomes which received a pulse of Na^+-free Krebs is shown with open circles and that in synaptosomes which recieved a pulse of 30 mM Na^+ is shown with filled circles. Efflux is expressed as the percentage of tissue $^{45}Ca^{2+}$ released per fraction. Results are given as the mean ± S.E. of five experiments. Where standard error bars are not shown they fall within the symbol.

Discussion

The results of this study confirm that decreasing extracellular Na^+ can cause the release of [^{3}H]-5-HT from superfused synaptosomes. Furthermore, there appears to be a good correlation between the reduction in extracellular Na^+ and the size of the response. However, the study failed to provide a clear indication of the mechanism by which the release occurred. The release did not appear to involve the plasma membrane 5-HT carrier except in conditions in which the availability of cytosolic 5-HT was increased by inhibiting monoamine oxidase. The experiments also showed that release was unlikely to have occurred as a result of an increase in cytosolic Ca^{2+} caused by changes in Na^+-dependent transmembrane Ca^{2+} fluxes. For example, the release of [^{3}H]-5-HT caused by Na^+ depletion was either independent of, or inversely related to, extracellular Ca^{2+} depending on the extent to which the extracellular Na^+ was reduced, and changes in $^{45}Ca^{2+}$ efflux caused by Na^+ depletion at different levels of extracellular Ca^{2+} did not correlate completely with the changes in 5-HT release.

These results suggest that the release of 5-HT caused by Na^+ depletion in the absence of monoamine oxidase inhibitors is not a simple process of carrier-mediated or Ca^{2+}-mediated efflux. However, morphological studies of tissue exposed to Na^+-free conditions do show changes consistent with the activation of the physiological release process [21]. On the assumption that the release process requires either the fusion of vesicles, or the movement of intramembranous proteins, to form transmitter efflux routes, it may be proposed that an

increase in membrane fluidity should lead to an increase in the ease with which such events could take place. This suggestion assumes that some form of spontaneous efflux occurs in CNS neurones which is similar to that which gives rise to miniature end plate potentials (m.e.p.p.s) in the neuromuscular junction. The frequency of these random events can be increased by a number of experimental procedures, the most well documented being the Ca^{2+}-dependent depolarisation-induced increase in m.e.p.p. frequency referred to as asynchronous release [22]. It has also been shown that m.e.p.p. frequency can be increased in a Ca^{2+}-independent manner by increasing the osmolarity of the extracellular fluid [23]. In the experiments reported here, the extracellular fluids used were isosmotic. Consequently such an effect could not account for the observations made in this study. It is clear however, that extracellular Ca^{2+} is not essential for increasing the spontaneous efflux of transmitter under all experimental conditions. Thus it is proposed that the release of 5-HT in response to extracellular Na^+ depletion is mediated by some form of enhanced spontaneous efflux which may be caused by an increase in membrane fluidity, and which is not dependent upon extracellular Ca^{2+}.

A number of the procedures used in these experiments would be expected to lead to an increase in membrane fluidity. For example, decreasing the Na^+ concentration or the ionic strength of the extracellular medium would reduce the screening of fixed negative charges on the external surface of the membrane and exert such an effect. The observation that decreasing extracellular Na^+ by either sucrose or choline substitution produced essentially equivalent responses would argue against changes in ionic strength or the influence of electrostatic forces on the screening of fixed negative charges being involved in these effects. If the effects are due to changes in screening, it is more likely that they are mediated by reducing some form of binding of Na^+ to the membrane which cannot be substituted by choline, but which may be antagonised by Ca^{2+}. If this is the case, a possible explanation may be offered for both the greater release seen with greater Na^+ reduction and for the differences in the effects of Ca^{2+} on 5-HT release at different degrees of Na^+ reduction. Although Ca^{2+} being divalent will have a greater influence in screening surface charges, its effects may be masked by the greater concentration of Na^+ when the latter is only reduced to 30 mM. In contrast, when Na^+ is reduced to zero, the screening effects of Ca^{2+} assume a greater significance such that the release of 5-HT increases as extracellular Ca^{2+} levels are decreased and is maximal in Ca^{2+}-free conditions. It is, however, difficult to quantify the actual contribution made by a mixture of monovalent and divalent cations in the screening of the negative charges on the surface of a dynamic cell membrane whose charge density may be constantly changing. Consequently this explanation must be considered as essentially speculative, and other forms of Na^+-Ca^{2+} antagonism which cannot be elucidated at this stage may be responsible for the observations made in this study. In this respect, it is interesting that Na^+-Ca^{2+} antagonism in transmitter release has been clearly shown at the neuromuscular junction under more physiological conditions than those used in this study [24].

In the final analysis, the results of this study suggest that the mechanism by which 5-HT efflux from superfused synaptosomes is stimulated by extracellular Na^+ depletion is not a simple process of carrier-mediated or Ca^{2+}-mediated release. It is more likely that release occurs as a result of non-specific changes in the behaviour of the plasma membrane which arise as a consequence of the ionic manipulations made when modifying extracellular Na^+. In the absence of a clear explanation of these effects, the release of 5-HT induced by Na^+ depletion, although interesting, is unlikely to be a technique of great value in elucidating the mechanism of transmitter release and its modulation.

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