INFLUENCES OF VERAPAMIL, X-537A, A-23187 AND ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE ON RELEASE OF 5-HYDROXYTRYPTAMINE FROM RAT BRAIN SLICES

Hiroko MURAKAMI, Eiko KAJI and Tomio SEGAWA
Department of Pharmacology, Institute of Pharmaceutical Sciences, Hiroshima University, School of Medicine, Hiroshima 734, Japan

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Abstract—Influences of verapamil, X-537A, A-23187 and cyclic-AMP on the release of [3H]-5-HT taken up into rat brain slices, were examined. Incubation with 40 mM KCl induced tritium release which was dependent on the presence of Ca²⁺. Verapamil, which blocks Ca²⁺ influx in excitable tissues, decreased potassium-induced release of 5-HT. Tritium release induced by ionophore X-537A was not dependent on extracellular Ca²⁺ while that induced by A-23187 required Ca²⁺. Cyclic-AMP, dibutyryl cyclic-AMP and theophylline did not significantly stimulate 5-HT release either in the presence or absence of Ca²⁺.

In a previous study from this laboratory we showed that high-potassium induced [3H]-5-hydroxytryptamine ([3H]-5-HT) release from rat brain synaptosomes was not affected when the amount of Ca²⁺ in the medium was decreased by 50% of the normal but such was completely abolished in Ca²⁺-free medium in the presence of EGTA (1). Our explanation is that although potassium-induced release of 5-HT is entirely dependent on Ca²⁺, only a small amount of Ca²⁺ in the medium is required to be transported into nerve terminals and intra- or extracellularly bound endogenous Ca²⁺ can be utilized for potassium-induced release of 5-HT.

Verapamil or its methyl-derivative D-600 is a drug that blocks Ca²⁺ influx in excitable tissue (2-4). X-537A and A-23187, which are termed ionophores, have been reported to bind Ca²⁺ and transport it across lipid membrane (5-7). It was therefore considered that these agents may be useful tools to obtain further information on the role of Ca²⁺. In addition, the effect of adenosine 3’,5’-cyclic monophosphate (cyclic-AMP) on high potassium-induced release of 5-HT was studied, since there is evidence suggesting that this nucleotide can increase Ca²⁺ permeability (8, 9) and facilitate the release of transmitters.

MATERIALS AND METHODS

The following drugs were generously donated: Verapamil (Eisei Pharmaceutical Co., Ltd., Tokyo, Japan); X-537A (Nippon Roche Research Center, Kamakura, Japan); A-23187 (Lilly Research Lab., Indianapolis, Ind.). 5-Hydroxy[G-3H]tryptamine creatinine sulphate ([3H]-5-HT) (500 mCi/m mole) was obtained from the Radiochemical Centre, Amersham. Adenosine 3’,5’-cyclic monophosphate and dibutyryl adenosine 3’,5’-cyclic monophosphate (dibutyryl cyclic-AMP) were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo,
Japan); Theophylline was from Katayama Chemical Industry Ltd. (Osaka, Japan).

**Uptake of [3H]-5-HT**

Male Wistar rats weighing from 100 to 140 g were decapitated and the whole brain was rapidly removed and placed on wet (Krebs-Ringer bicarbonate solution) filter paper on ice. A coronal section of the brain was made about 4 mm from the anterior pole of the cerebral hemispheres and slices (diameter 3 mm, thick 0.4 to 0.5 mm, wet weight approx. 20 mg) of cerebral cortex and neostriatum were prepared from the posterior portion with a razor blade. These slices were suspended in O₂-saturated Krebs-Ringer bicarbonate solution containing $2 \times 10^{-7}$ M pheniprazine (a monoamine oxidase inhibitor), 0.02% ascorbic acid and [3H]-5-HT (final concentration of $10^{-7}$ M) and were incubated at 37°C for 30 min.

**Release of [3H]-5-HT**

After rinsing several times in fresh medium, the slices were suspended in 0.5 ml of the medium and again incubated at 37°C. The medium was renewed every 5 min for 30 min, thereafter they were incubated in the medium containing 40 mM KCl (40 mM NaCl was omitted) for 5 min. Unless otherwise stated, the agents to be tested were dissolved in 0.015M Tris-buffered saline (pH 7.4) and were diluted with Krebs-Ringer bicarbonate solution when tested. In each experiment, the fraction for every 5 min was collected in a scintillation vial containing 10 ml Bray's solution and the radioactivity was determined in a model 3320 Packard Tri-Carb liquid scintillation spectrometer and corrected for efficiency by external standardization. Counting efficiency was approx. 20%.

Release of [3H]-5-HT was expressed as dpm. The potassium-induced [3H]-5-HT release was also expressed as percent of spontaneous tritium release during 5 min before high potassium.

A-23187 and X-537A were dissolved in DMSO and an approximate dilution was made in Krebs-Ringer bicarbonate solution so that the concentration of DMSO did not exceed 1%.

The Krebs-Ringer bicarbonate solution was composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.13 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose.

**RESULTS**

**Potassium-induced release and Ca²⁺ dependency**

As is shown in Fig. 1, incubation of brain slices in the medium containing 40 mM KCl for 5 min produced a striking increase in [3H]-5-HT release, the efflux being increased to 4.2 times the control. In preliminary experiments, the release of tritium was examined for the presence of metabolite of [3H]-5-HT and it was found that the proportion of unchanged [3H]-5-HT was 63.5% of the total release of radioactivity in spontaneous release, while in the potassium-induced release such was 82.1%.

As is shown in Table 1, pre-incubation with Ca²⁺-free medium for 5 min failed to modify both the potassium-induced and spontaneous releases. However, when the slices were incubated with Ca²⁺-free medium for 30 min or with Ca²⁺-free medium in the presence of EGTA (2.5 mM) for 5 min, the potassium-induced release was significantly attenuated.
while spontaneous release was markedly but not significantly increased in Ca^{2+}-free and EGTA-containing medium.

**Effect of verapamil**

The results are shown in Table 2. Verapamil was introduced into the incubation medium.
30 min before potassium stimulation. At $10^{-6}$ M, verapamil did not produce any significant change in either spontaneous or potassium-induced [H]-5-HT release. At $10^{-7}$ and $10^{-4}$ M, however, verapamil induced an increase in spontaneous release and a decrease in potassium-induced release.

**Effect of X-537A and A-23187**

When the slices were exposed to the medium containing either X-537A or A-23187, there was marked increase in [H]-5-HT release. Fig. 2 shows the time course of the effect of these ionophores. The rate of increased release by X-537A ($10^{-5}$ M) was rapid. Thus, within 5 min there was approx. $5 \times 10^8$ dpm of tritium release, thereafter the release increased.

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**TABLE 3. Effect of Ca$^{2+}$-deficiency on X-537A and A-23187-induced release of [H]-5-HT from rat brain slices**

| Drugs      | (M) | Medium          | Ca$^{2+}$-free |
|------------|-----|-----------------|---------------|
| Control    |     | Normal          | 1184±107 (8)  | 1295±58 (8)  |
| (1% DMSO)  |     |                 |               |
| X-537A     | $10^{-5}$ | 5493±448 (6)    | 6155±324 (6)  |
|            | $10^{-4}$ | 5189±661 (5)    | 5815±492 (5)  |
| A-23187    | $4 \times 10^{-5}$ | 591±150 (5)    | 247±163 (5)   |
|            | $10^{-4}$ | 798±124 (6)     | 327±92 (6)*   |
|            | $2 \times 10^{-4}$ | 1155±225 (6)   | 717±158 (6)   |

Rat brain slices, pre-loaded with [H]-5-HT were incubated at 37°C for 10 min. Ionophore-induced release was expressed as the difference between dpm released by 1% DMSO and by ionophore. Values are mean±S.E.M. of number of determinations indicated in parentheses.

* Significantly different at $P<0.02$ from the values in normal medium.
almost linearly for about 20 min, after which the rate somewhat declined. A-23187 was less potent than X-537A in releasing [3H]-5-HT from brain slices (Fig. 2). Preliminary experiments indicated that when the slices were incubated with 40 mM KCl for more than 5 min there was no further increase in tritium release.

The influence of omission of Ca\(^{2+}\) from the medium on the [3H]-5-HT release induced by ionophore was examined, using 10 min incubation with ionophore. In this experiment, ionophore-induced release was expressed as the difference between the radioactivity release by 1% DMSO and by and by ionophore in DMSO (Table 3). A-23187 \((4 \times 10^{-4} - 2 \times 10^{-4} \text{ M})\) increased the tritium release in Ca\(^{2+}\)-normal medium, depending on the concentration employed. The effect was considerably reduced when the experiment was performed under Ca\(^{2+}\)-free condition (significantly different at \(10^{-4} \text{ M A-23187}\)). X-537A, at \(10^{-6} \text{ M}\), remarkably increased the tritium release in normal medium but no further increase in release was observed at \(10^{-5} \text{ M}\). The omission of Ca\(^{2+}\) from the medium failed to attenuate the effect of X-537A, rather such was increased.

**Effect of cyclic-AMP**

The results are summarized in Table 4. Cyclic-AMP \((10^{-4} \text{ M})\) did not affect either spontaneous or potassium-induced [3H]-5-HT release. Dibutylryl cyclic-AMP, at the same concentration, slightly but not significantly increased potassium-induced release without affecting spontaneous release. Theophylline \((10^{-3} \text{ M})\) had no effect on tritium release. When the slices were incubated in the medium containing cyclic-AMP \((10^{-4} \text{ M})\) and theophylline \((10^{-3} \text{ M})\), there was no change in either spontaneous or potassium-induced release. Cyclic-AMP \((10^{-4} \text{ M})\) had no effect on tritium release in Ca\(^{2+}\)-free medium.

**Table 4. Effect of cyclic-AMP, dibutylryl cyclic-AMP and theophylline on [3H]-5-HT release from rat brain slices**

| Medium    | Drugs     | Concentration (M) | Spontaneous (dpm) | Release | Potassium-induced Release (dpm) | Potassium-induced Spontaneous (%) |
|-----------|-----------|-------------------|-------------------|---------|---------------------------------|----------------------------------|
| Normal    | Control   |                   | 691 ± 25 (6)      | 2895 ± 125 (6) | 420 ± 12 (6)                    |
|           | Cyclic-AMP| 10^{-4}           | 743 ± 30 (6)      | 2906 ± 199 (6) | 389 ± 14 (6)                    |
|           | Control   | 10^{-4}           | 706 ± 109 (8)     | 2299 ± 364 (8) | 347 ± 41 (8)                    |
|           | Dibutylryl cyclic-AMP | 10^{-4} | 719 ± 119 (8) | 2625 ± 357 (8) | 384 ± 23 (8)                    |
|           | Control   | 10^{-2}           | 694 ± 33 (6)      | 2661 ± 87 (6)  | 387 ± 27 (6)                    |
|           | Theophylline | 10^{-2}         | 694 ± 75 (6)      | 2586 ± 96 (6)  | 389 ± 33 (6)                    |
|           | Control   | 10^{-2}           | 640 ± 60 (6)      | 2056 ± 297 (6) | 346 ± 55 (6)                    |
|           | Cyclic-AMP | 10^{-4}           | 565 ± 54 (6)      | 1832 ± 140 (6) | 343 ± 46 (6)                    |
|           | Theophylline | 10^{-3}         | 556 ± 53 (6)      | 855 ± 40 (6)   | 156 ± 10 (6)                    |
| Ca\(^{2+}\)-free | Control   | 10^{-4}           | 576 ± 45 (6)      | 847 ± 25 (6)   | 150 ± 8 (6)                     |
|           | Cyclic-AMP | 10^{-4}           | 1115 ± 194 (6)    | 1946 ± 325 (6) | 179 ± 15 (6)                    |
|           | Dibutylryl | 10^{-4}           | 964 ± 134 (8)     | 1742 ± 256 (8) | 179 ± 7 (8)                     |

Rat brain slices, pre-loaded with [3H]-5-HT were incubated with drugs at 37°C for 5 min. Values are mean ± S.E.M. of number of determinations indicated in parentheses.
As in the case of other putative transmitters, depolarization by high potassium effectively released [3H]-5-HT from brain slices. Furthermore, potassium-induced release appeared to be dependent on the presence of Ca\(^{2+}\). However, 5-HT release could be abolished only when the preparation was incubated with Ca\(^{2+}\)-free medium for 30 min or with Ca\(^{2+}\)-free medium in the presence of EGTA for 5 min. These results are generally coincident with our previous observations with synaptosomes (1).

D-600, an analogue of verapamil, was found to inhibit both vasopressin and oxytocin release from the neurohypophysis (4, 10), and catecholamine from the adrenal medulla (11). Our result shows that verapamil, at a concentration which is known to be effective in blocking Ca\(^{2+}\) entry, decreased potassium-induced 5-HT release. Therefore, influx of Ca\(^{2+}\) into the nerve cell is a necessary event for release of [3H]-5-HT.

The antibiotics ionophores X-537A and A-23187 are known to form a lipophilic complex with monovalent and/or divalent cations, thereby increasing their permeability of biological membrane. Ionophores have also been shown to induce catecholamine release from peripheral adrenergic neurons (12), brain synaptosomes (13, 14) and adrenal medulla (15) ACh release from brain slices (16) and ATP from platelets (17). The results presented here on release of [3H]-5-HT from brain slices are consistent with these observations. X-537A released approximately 4 times as much tritium as did A-23187, though the concentration was only one fourth that of A-23187. The release of [3H]-5-HT from slices by A-23187 was markedly reduced when Ca\(^{2+}\) was omitted. These results are similar to those observed in the case of catecholamine release (12, 13). On the other hand, X-537A action did not require Ca\(^{2+}\) in the medium. X-537A differs from A-23187 in that it has relatively little selectivity for divalent cations (18). It forms lipophilic complexes with monovalent cations such as Na\(^{+}\), K\(^{+}\), Cs\(^{+}\) and H\(^{+}\). However, the possibility that X-537A depolarized the preparation by increasing Na\(^{+}\) entry is excluded, since the effect did not require extracellular Ca\(^{2+}\). Though the possibility that X-537A may act as Ca-ionophore in the presence of a trace amount of external Ca\(^{2+}\) cannot be excluded, there is also the possibility that X-537A enters the cell thus causing Ca\(^{2+}\) to be released from intracellular stores (19) or X-537A releases 5-HT by an indirect, reserpine like mechanism (12).

Theophylline, cyclic-AMP and dibutyryl cyclic-AMP can stimulate catecholamine release from the adrenal medulla in the absence of external Ca\(^{2+}\) (20). Similarly, independently of external Ca\(^{2+}\), phosphodiesterase inhibitors, aminophylline or caffeine can stimulate catecholamine release from the adrenal medulla (21, 22) and dibutyryl cyclic-AMP and theophylline can release norepinephrine from sympathetic nerves (23). On the basis of these observations, it has been suggested that cyclic-AMP may stimulate release by mobilizing intracellular, bound Ca\(^{2+}\). In the present experiment, it was shown that cyclic-AMP, dibutyryl cyclic-AMP and theophylline did not significantly stimulate [3H]-5-HT release either in the presence or absence of external Ca\(^{2+}\). It would thus appear that in serotonergic nerve terminals, cyclic-AMP is not involved in mobilizing intracellular, bound Ca\(^{2+}\).

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