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

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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 Ca^{2+} . Verapamil, which blocks Ca^{2+} influx in excitable tissues, decreased potassium-induced release of 5-HT. Tritium release induced by ionophore X-537A was not dependent on extracellular Ca^{2+} while that induced by A-23187 required Ca^{2+} . Cyclic-AMP, dibutyryl cyclic-AMP and theophylline did not significantly stimulate 5-HT release either in the presence or absence of Ca^{2+} .

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 Ca^{2+} in the medium was decreased by 50% of the normal but such was completely abolished in Ca^{2+} -free medium in the presence of EGTA (1). Our explanation is that although potassium-induced release of 5-HT is entirely dependent on Ca^{2+} , only a small amount of Ca^{2+} in the medium is required to be transported into nerve terminals and intra- or extracellularly bound endogenous Ca^{2+} can be utilized for potassium-induced release of 5-HT.

Verapamil or its methyl-derivative D-600 is a drug that blocks Ca^{2+} influx in excitable tissue (2-4). X-537A and A-23187, which are termed ionophores, have been reported to bind Ca^{2+} 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 Ca^{2+} . 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 Ca^{2+} 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/mmol) 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 2×10^{-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

FIG. 1. Release of [^3H]-5-HT from rat brain slices. Rat brain slices, pre-loaded with [^3H]-5-HT were incubated at 37°C . Release is expressed as dpm during 5 min incubation.

TABLE 1. Effect of Ca^{2+} -deficiency on [^3H]-5-HT release from rat brain slices

Medium	Condition Incubation time (min)	Spontaneous (dpm)	Release Potassium-induced (dpm)	Potassium-induced Spontaneous (%)
Normal	5	639 ± 92 (7)	2640 ± 334 (7)	420.3 ± 17.0 (7)
Ca^{2+} -free	5	583 ± 77 (3)	2548 ± 542 (3)	429.0 ± 22.4 (3)
Ca^{2+} -free	20	542 ± 72 (4)	1062 ± 248 (4)*	191.3 ± 20.7 (4)*
Ca^{2+} -free + EGTA (2.5 mM)	5	872 ± 242 (4)	1098 ± 256 (4)*	129.6 ± 5.1 (4)*

Rat brain slices, pre-loaded with [^3H]-5-HT were incubated at 37°C . Spontaneous release is dpm during 5 min incubation before addition of high potassium. Potassium-induced release is dpm during 5 min incubation with 40 mM KCl. Values are mean $\pm$ S.E.M. of number of determinations indicated in parentheses. * $P < 0.01$.

TABLE 2. Effect of verapamil on [^3H]-5-HT release from rat brain slices

Concentration of verapamil (M)	Spontaneous (dpm)	Release Potassium-induced (dpm)	Potassium-induced Spontaneous (%)
Control	894 ± 85	2931 ± 305	345.3 ± 3.1
10^{-6}	900 ± 96	3122 ± 389	347.1 ± 22.7
10^{-5}	1037 ± 139	2463 ± 330	$238.5 \pm 21.8^*$
10^{-4}	$1342 \pm 91^*$	$1096 \pm 73^*$	$81.9 \pm 2.4^*$

Rat brain slices, pre-loaded with [^3H]-5-HT were incubated at 37°C . Slices were incubated with verapamil for 30 min before addition of high potassium. Release is expressed as dpm during 5 min incubation. Values are mean $\pm$ S.E.M. of 5 determinations. * $P < 0.01$.

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 [3 H]-5-HT release. At 10^{-5} 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 [3 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. 5×10^3 dpm of tritium release, thereafter the release increased

FIG. 2. Time course of X-537- and A-23187-induced [3 H]-5-HT release from rat brain slices. Rat brain slices, pre-loaded with [3 H]-5-HT were incubated at 37°C with ionophores. Values are mean $\pm$ S.E.M. of 5 determinations.

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

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

Rat brain slices, pre-loaded with [3 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 $\pm$ 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 (4×10^{-5} – 2×10^{-4} 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} M A-23187). X-537A, at 10^{-6} M, remarkably increased the tritium release in normal medium but no further increase in release was observed at 10^{-5} 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} M) did not affect either spontaneous or potassium-induced [^3H]-5-HT release. Dibutyl cyclic-AMP, at the same concentration, slightly but not significantly increased potassium-induced release without affecting spontaneous release. Theophylline (10^{-3} M) had no effect on tritium release. When the slices were incubated in the medium containing cyclic-AMP (10^{-4} M) and theophylline (10^{-3} M), there was no change in either spontaneous or potassium-induced release. Cyclic-AMP (10^{-4} M) had no effect on tritium release in Ca^{2+} -free medium.

TABLE 4. Effect of cyclic-AMP, dibutyl cyclic-AMP and theophylline on [^3H]-5-HT release from rat brain slices

Medium	Drugs	Concentration (M)	Spontaneous (dpm)	Release Potassium-induced (dpm)	Potassium-induced Spontaneous (%)
Normal	Control		691 $\pm$ 25 (6)	2895 $\pm$ 125 (6)	420 $\pm$ 16 (6)
	Cyclic-AMP	10^{-4}	743 $\pm$ 30 (6)	2906 $\pm$ 199 (6)	389 $\pm$ 14 (6)
	Control		706 $\pm$ 109 (8)	2299 $\pm$ 364 (8)	347 $\pm$ 41 (8)
	Dibutyl cyclic-AMP	10^{-4}	719 $\pm$ 119 (8)	2625 $\pm$ 357 (8)	384 $\pm$ 23 (8)
	Control		694 $\pm$ 33 (6)	2661 $\pm$ 87 (6)	387 $\pm$ 27 (6)
	Theophylline	10^{-3}	694 $\pm$ 75 (6)	2586 $\pm$ 96 (6)	389 $\pm$ 33 (6)
	Control		640 $\pm$ 60 (6)	2056 $\pm$ 297 (6)	346 $\pm$ 55 (6)
	Cyclic-AMP	10^{-4}	565 $\pm$ 54 (6)	1832 $\pm$ 140 (6)	343 $\pm$ 46 (6)
Ca^{2+} -free	Theophylline	10^{-3}			
	Control		564 $\pm$ 53 (6)	853 $\pm$ 40 (6)	156 $\pm$ 10 (6)
	Cyclic-AMP	10^{-4}	576 $\pm$ 45 (6)	847 $\pm$ 25 (6)	150 $\pm$ 8 (6)
	Control		1115 $\pm$ 194 (6)	1946 $\pm$ 325 (6)	179 $\pm$ 15 (6)
	Dibutyl	10^{-4}	964 $\pm$ 134 (8)	1742 $\pm$ 256 (8)	179 $\pm$ 7 (8)

Rat brain slices, pre-loaded with [^3H]-5-HT were incubated with drugs at 37°C for 5 min. Values are mean $\pm$ S.E.M. of number of determinations indicated in parentheses.

DISCUSSION

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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