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FACTORS AFFECTING THE RELEASE OF PURINES FROM MOUSE CEREBRAL CORTEX: POTASSIUM REMOVAL AND METABOLIC INHIBITORS

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Abstract—The release of purines from slices of mouse cerebral cortex was examined after pre-incubation with [^{3}H]adenine or [^{3}H]adenosine. Ouabain and perfusion with K^+-free medium evoked a Ca^{2+}-dependent-release, although low K^+ solutions reduced release evoked by ouabain. The metabolic inhibitors fluoride, azide and *p*-hydroxymercuribenzoate also induced a large purine release which was Ca^{2+}-dependent but which was increased by methylxanthines and decreased by dipyridamole and hexobendine. It is suggested that purine release is not related to (Na^+, K^+) ATPase inhibition, and that the mechanism of release by metabolic inhibitors is different from the mechanism of ouabain induced release.

It has been suggested that purine derivatives, particularly adenosine and ATP may function as neurotransmitters [1, 2] or neuromodulators [3, 4] in the nervous system. Evidence for such a role has included the demonstration of release from cerebral slices [5–7] or synaptosomes [2, 9] but the release obtained by conventional depolarising agents such as K^+ is far less than is produced by ouabain or veratridine [5, 6, 8]. This contrasts with the substantial release of classical transmitters such as GABA or amines, which are produced by high K^+ concentrations [10].

Some properties of ouabain-induced purine release have recently been examined, including in particular the reduction of release by methylxanthines [11]. As it has been reported that some metabolic inhibitors can release purines from brain [12] we have now investigated some of the properties of that release for comparison.

MATERIALS AND METHODS

Male TO or MF1 mice were killed by stunning and cervical dislocation. The skull was opened, the dorsal lying area of cerebral cortex removed and placed immediately into a solution at 0° of the following composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$ $2H_2O$,2.4; $NaHCO_3$, 26; glucose, 10. The tissue was transferred to a McIlwain tissue chopper and slices cut at a thickness of 400 μm. Three to six slices were placed on a nylon mesh in glass incubation chambers of 2.5 ml capacity containing bathing fluid at 37° gassed with 95% O_2/5% CO_2. After 20 min incubation, 5 to 20 μCi of [^{3}H]adenine or [^{3}H]adenosine (sp. act. 20 Ci/mmole) were added (Radiochemical Centre, Amersham) and incubation continued for a further 20 min. [^{3}H]Adenosine was used in the large majority of these experiments, [^{3}H]adenine only in a few of our earliest control and ouabain release experiments. Although results were similar using either precursor, the use of [^{3}H]adenosine yielded higher tissue and release counts, and thus improved counting accuracy. The incubation solution was then withdrawn and replaced with a further 2.5 ml of gassed medium at 37° every 2 min [8]. Two experiments were run in parallel using tissue from the same animal. Following the incubation with labelled purines, dipyridamole (10^{-5} M) was included in the incubating medium throughout most experiments in order to prevent the reuptake of released adenosine [1, 13]. In general, however, the omission of dipyridamole had little net effect on the quantity of label released.

After an initial washout period of 30 min the samples were retained for counting by liquid scintillation spectrometry. Release was usually evoked by ouabain 10^{-4} M, added to the incubating medium for a period of 20 min.

In order to compare the release of purines with the release of a conventional neurotransmitter, a number of experiments have been performed using [^{14}C]-γ-aminobutyric acid (GABA) of S.a 220 mCi/mmole. Initial incubations were carried out in 1 μCi of [^{14}C]-GABA, and the subsequent fluid exchanges made in solution containing 10^{-4} M amino-oxy-acetic acid to inhibit GABA transaminase.

Experiments were also performed using a conventional continuous superfusion process from a pump providing a nonpulsatile flow rate of approximately 0.5 ml/min.

Previous groups have shown that the material released from brain slices after incubation with labelled adenine or adenosine consists partly of adenosine together with inosine and hypoxanthine.

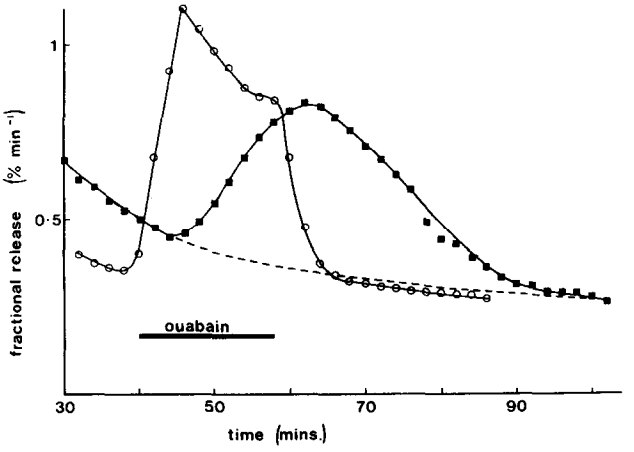

Fig. 1. Graphs illustrating the time course of release of ^{3}H-labelled purines (■) and [^{14}C]-GABA (○) from cerebral cortex slices resulting from the presence of ouabain, 0.1 mM in the medium. The dotted line on the curve of purine release indicates the way in which the baseline was projected in order to quantify the release increment attributable to ouabain. Results are from a typical experiment; means and standard errors are shown in Table 1. On the ordinate, release in each 2 min fraction is expressed as a percentage of the radioactivity remaining in the tissue at the end of the experiment. The time axis begins after 30 min washing following incubation with the label.

We have confirmed this by subjecting samples of the bathing medium to thin layer chromatography on silica gel coated sheets (Merck) containing a fluorescent additive (SGF_{254}) using the solvent system of *n*-butanol: ethylacetate: methanol: ammonia (7:4:3:4 v/V). Purine spots were located under UV light at 243 nm, scraped into vials, eluted with 0.1 M HCl for 1 hr and counted by liquid scintillation spectrometry. The compounds released both in the resting state and the ouabain stimulated state were present in the proportions adenosine 20%; inosine 45%; hypoxanthine 35%. These ratios are very similar to those reported by Lewin and Bleck [14].

At the end of each experiment the slices were solubilised in scintillation fluid and the final tissue radioactivity assessed by liquid scintillation spectrometry. Results are presented as the radiolabel released in each 2 min sample expressed as a fraction of the final tissue content.

In order to assess the release of label induced by drugs, a graph of release against time was first constructed. The baseline release which would have occurred in the absence of drug was then estimated by interpolating the curve as illustrated in Fig. 1.

In both drug and drug-free control experiments the release of purines had reached a plateau after 30 min and a value for baseline release was therefore calculated as the mean release occurring in samples 15 to 20 (30 to 40 min) (Table 1).

The release of label induced by drugs was calcu-

Table 1. Release of purines from slices of mouse cerebral cortex

Stimulus	Solution	Peak purine release (% min^{-1})	% Change
None	normal	0.32 ± 0.01(82)*‡	–
Ouabain 0.1 mM	normal	0.66 ± 0.12(6)	106†
Ouabain 1 mM	normal	0.83 ± 0.10(4)	159†
Ouabain 0.1 mM	0Na^{+} (choline)	0.39 ± 0.06(4)	22
Ouabain 0.1 mM	0Ca^{2+} (+EGTA)	0.37 ± 0.06(4)	16

* Results are expressed as the ±S.E. of the mean where 4 or more experiments were performed. The number of experiments is shown in parentheses.

† Significantly different from baseline at $P < 0.05$ using Student's *t*-test.

‡ As we have not determined the specific activity of the labelled compounds present in or released by the tissue we cannot accurately convert this fractional release to absolute molar quantities. However, this release from control experiments corresponds to approximately 8.6 picomols of ^{3}H-labelled compounds/g cortex/min. If we take the total adenine nucleotide content as 2.8 μmoles/g fresh brain [22] and assume the tissue radioactivity is distributed evenly throughout the adenine nucleotide pools, the release then corresponds to approximately 8.9 nmoles, of adenine compounds/g cortex/min. These two estimates therefore provide approximate lower and upper limits respectively for the absolute level of release.

lated both in terms of the peak release, and in terms of the area between the drug induced release curve and the interpolated baseline curve. The two methods of analysis yielded comparable results, but as the presentation of data in terms of peak release permits a readier comparison with baseline values, the peak release was used in compiling the results as presented here.

RESULTS

In concentrations of 0.1 mM and 1 mM ouabain evoked a release of purines (Table 1), though with a relatively slow time course, illustrated in Fig. 1. Removal of Na^+, and its replacement with choline, or the removal of Ca^{2+} and inclusion of EGTA, (0.5 mM) reduced the ouabain evoked release (Table 1).

In marked contrast to this behaviour, a release of GABA was evoked far more rapidly by ouabain, the release usually declining before the end of the ouabain pulse [11].

Potassium removal

The removal of K^+ from the extracellular fluid produced a small decrease of purine release which then tended to recover towards or above the projected normal curve (Fig. 2). In 7 of 10 experiments, however, a substantial increment of release was seen when the K^+ levels were restored to normal (Fig. 2; Table 2). Neither of these changes were obtained when the experiment was performed in Ca^{2+}-free medium containing EGTA 0.5 mM. It will again be seen from Fig. 2 that the release of GABA followed a much more rapid time course on lowering the extracellular K^+.

Figure 3 illustrates the effect of K^+-free medium, introduced during the release phase induced by ouabain. It was a consistent finding (4 experiments) that the removal of K^+ caused an immediate reduction of evoked release, whereas release normally increased throughout the ouabain period, and indeed often reached a maximum some minutes after ouabain was removed (Fig. 1).

In 16 experiments two periods of low K^+ were used, separated by an interval of 12 to 20 minutes. In 10 of these experiments the cortical slices were bathed in the normal solution for the period between the two low K^+ solutions, but in 6 the intervening period was spent in a solution containing no Na^+

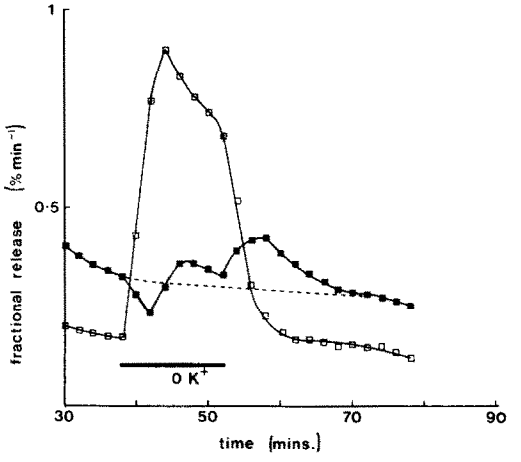

Fig. 2. Graphs illustrating the time course of release from cortex slices of [^{3}H]purines (■) and [^{14}C]-GABA (□), produced by a potassium-free solution. Note the very rapid release of GABA, compared with an initially decreased release of purines, and secondary increment of purine release when normal K^+ levels are restored. Release on the ordinate is expressed as a fraction of the radioactivity remaining in the tissue at the end of the experiment. The time axis begins after 30 min washing of the labelled tissue.

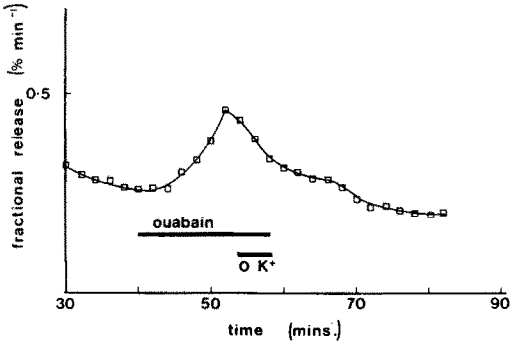

Fig. 3. graph showing the combined effect of ouabain and a K^+-free solution on the release of [^{3}H]purines from cortex slices. Note that the release produced by ouabain is inhibited by the use of a K^+-free medium. The release curve should be compared with that of Fig. 1, where it will be seen that the peak release normally occurred only after ending the ouabain stimulation. Axes as in Fig. 1.

Table 2. Release of purine from slices of mouse cerebral cortex

Stimulus	Solution	Peak of purine release (% min^{-1})*	% Change
None	normal	0.32 ± 0.01(82)	–
0K$^+$	normal	0.78 ± 0.10(10)	143‡
0K$^+$	Ca^{2+}-free	0.44 ± 0.02(4)	37†
0K$^+$ (1)	normal	0.72 ± 0.08(10)	125†
0K$^+$ (2)		0.63 ± 0.06(10)	97†
0K$^+$ (1)	Na^+-free	0.70 ± 0.11(6)	119†
0K$^+$ (2)		0.44 ± 0.09(16)	37†

* Mean ± 1 S.E. of the mean (n).
† Significantly different from baseline at $P < 0.05$ using Student's t-test.

replaced with choline as the bicarbonate and chloride). When the standard solution was being used, the second period of OK^+ caused an increase of release comparable with that of the first stimulus (Table 2). When $0Na^+$ solution was present between the $0K^+$ stimuli, very little release of label was seen either during or following the second $0K^+$ stimulus.

Fluoride, azide and p-hydroxymercuribenzoate (pHMB)

The presence of 1 mM or 10 mM sodium fluoride in the incubation medium led to a release of purines. Release induced by 1 mM fluoride was comparable in amount with that produced by ouabain (0.1 mM), though fluoride evoked release was much more rapid in onset and, as with pHMB, decayed somewhat even during the continued presence of fluoride.

The inclusion in the bathing medium of two non-xanthine phosphodiesterase inhibitors, Ro 20–1724 and ICI 63, 197 at concentrations of 1 mM reduced slightly the fluoride evoked release (Table 3). Caffeine, at 10^{-3} M however, increased the release some 40 per cent.

At 10^{-5} M, dipyridamole reduced the fluoride-evoked release by almost 70 per cent (Table 3) and a comparable reduction accompanied the inclusion of hexobendine, 10^{-5} M.

Sodium azide, 5 mM and pHMB, 0.5 mM also proved able to evoke purine release. In both cases the release was enhanced by the inclusion of caffeine or IBMX at 1 mM (Table 3).

Release induced by all three of these agents, fluoride, azide and pHMB proved to be Ca^{2+}-dependent: the ommission of Ca^{2+} and inclusion of EGTA, 0.1 mM in the incubation medium greatly reduced evoked release, as did the inclusion of verapamil, 10^{-4} M (table 3).

Using labelled GABA instead of adenosine, it was found that pHMB produced a rapid release of the label, whereas fluoride, even at 100 mM produced no release at all.

DISCUSSION

Several of the findings presented above support our earlier conclusion that purine release is not related to activity of (Na^+, K^+) ATPase (E.C.3.5.1.3). Firstly ouabain, at a concentration of 10^{-3} M, produces a total inhibition of (Na^+, K^+) ATPase in cerebral synaptosomes [15], yet fluoride and pHMB, an inactivator of sulphydryl groups, produced a three-fold greater release of purines, and with a much shorter latency, than ouabain.

Secondly inhibition of (Na^+, K^+)ATPase produced by the removal of K^+ from the bathing medium elicited only a small release of purine, compared with the release of GABA, for example, as reported elsewhere [16]. Potassium removal also evokes a rapid release of acetylcholine at the neuromuscular junction [17] emphasising the considerable differences between purine release and conventional neurotransmitters.

Table 3. Release of purines from slices of mouse cerebral cortex

Stimulus	Concn (mM)	Conditions	Peak release (% min^{-1})	% Change
None	—	—	0.32 ± 0.01(82)*	—
Fluoride	10	—	1.41 ± 0.10(6)	341
Fluoride	10	$0Ca^{2+}$	0.56 ± 0.11(4)	75
Fluoride	10	Verapamil 0.1 mM	0.54 ± 0.14(4)	69
Fluoride	10	Ro20-1724 1 mM	1.30(2)	306
Fluoride	10	ICI 63, 197 1 mM	1.26(2)	294
Fluoride	10	Caffeine 1 mM	1.82 ± 0.16(4)	469
Fluoride	10	dipyridamole 10 μM	0.64 ± 0.13(4)	100
Fluoride	10	hexobendine 10 μM	0.59 ± 0.14(4)	84
Fluoride	1	—	0.64 ± 0.15(4)	100
Fluoride	1	$0Ca^{2+}$	0.41 ± 0.13(4)	28
Fluoride	1	Verapamil 0.1 mM	0.40(2)	25
Fluoride	1	Ro20-1724 1 mM	0.61(2)	91
Fluoride	1	ICI 63, 197 1 mM	0.66(2)	106
Fluoride	1	caffeine 1 mM	0.90 ± 0.11(4)	181
Fluoride	1	dipyridamole 10 μM	0.40 ± 0.10(4)	25
Fluoride	1	hexobendine 10 μM	0.42(2)	31
Azide	5	—	0.94 ± 0.15(4)	194
Azide	5	$0Ca^{2+}$	0.62(2)	94
Azide	5	Verapamil 0.1 mM	0.53(2)	66
Azide	5	caffeine 1 mM	1.19 ± 0.14(4)	272
Azide	5	IBMX 1 mM	1.28(2)	300
pHMB	0.5	—	1.38 ± 0.21(4)	331
pHMB	0.5	$0Ca^{2+}$	0.66 ± 0.13(4)	106
pHMB	0.5	verapamil 0.1 mM	0.54(2)	69
pHMB	0.5	caffeine 1 mM	1.64 ± 0.19(4)	412
pHMB	0.5	IBMX 1 mM	1.45(2)	353

* Mean ± S.E. of the mean (n) where 4 or more experiments were performed.

Finally, and of particular significance, is the observation that ouabain-evoked release was *reduced* by the use of low K^+ solutions. If ouabain induced release were due to inhibition of (Na^+, K^+)ATPase it might reasonably have been expected that the combination of procedures would increase purine release.

The question therefore remains as to the origin and mechanism of purine release. The experiments with two periods of $0K^+$ were prompted by the report of Jirounek *et al.* [18]. This group found a transient release of inorganic phosphate in $0K^+$ medium which was reproducible with a second such period only after incubation in medium of normal composition. Incubation in low Na^+ medium greatly reduced the release obtained by a second $0K^+$ stimulus. Clearly there may be some similarity between the observations of Jirounek *et al* [18] and those of the present study, implying possibly a common component in the mechanism of release of purines and phosphate. Certainly Ritchie and Straub [19] are of the opinion that much of the phosphate released from neurones originates from the breakdown of ATP during and following activity, and such a breakdown could well lead to increased levels of adenosine and its metabolites which would then diffuse out of the cells.

The use of fluoride, azide and pHMB was designed to examine the characteristics of the release evoked by metabolic inhibitors. Fluoride is known to inhibit several enzyme systems including (Na^+, K^+)ATPase [20] as does pHMB by virtue of its inactivating sulphydryl groups. Our demonstration that purine release can be evoked by these compounds therefore complements the report of Daval *et al* [12] which showed release evoked by cyanide, iodoacetate and FCCP (carbonyl cyanide-β-fluoromethoxy-phenylhydrazone), and the finding that purine efflux is increased during hypoxic conditions [5].

However, while the apparent Ca^{2+}-dependence of release represents a point of similarity between the release evoked by azide and fluoride and that induced by ouabain, there are marked differences in the properties of the release process. Thus ouabain-evoked release is greatly reduced by caffeine, IBMX and other methylxanthines, but not affected by dipyridamole or hexobendine [11]. Conversely, however, fluoride, azide and pHMB evoked release is increased by methylxanthines but diminished by dipyridamole and hexobendine. At present we have no explanation for these latter findings. As dipyridamole and hexobendine inhibit the uptake of adenosine into cells [1, 13] it would be expected that any effect they might have would be in the direction of an increased purine release, and their inhibition of release is therefore particularly baffling. However, reduction of purine release by dipyridamole from isolated desheathed vagus nerve has recently been described [21], so this observation is not unique to CNS tissue.

We suggest from the present results that ouabain-induced release of purines is not related to inhibition of (Na^+, K^+)ATPase, but involves a mechanism different from that causing release after metabolic interference. The data are not inconsistent with our previous proposal that ouabain-induced purine release results from the activation of adenylate cyclase and the consequent elevation of cyclic AMP levels [11].

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