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Sustained depolarisation induces changes in the extracellular concentrations of purine and pyrimidine nucleosides in the rat thalamus

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Abstract

ATP and adenosine are well-known neuroactive compounds. Other nucleotides and nucleosides may also be involved in different brain functions. This paper reports on extracellular concentrations of nucleobases and nucleosides (uracil, hypoxanthine, xanthine, uridine, 2′-deoxycytidine, 2′-deoxyuridine, inosine, guanosine, thymidine, adenosine) in response to sustained depolarisation, using in vivo brain microdialysis technique in the rat thalamus. High-potassium solution, the glutamate agonist kainate, and the Na^+/K^+ ATPase blocker ouabain were applied in the perfusate of microdialysis probes and induced release of various purine and pyrimidine nucleosides. All three types of depolarisation increased the level of hypoxanthine, uridine, inosine, guanosine and adenosine. The levels of measured deoxynucleosides (2′-deoxycytidine, 2′-deoxyuridine and thymidine) decreased or did not change, depending on the type of depolarisation. Kainate-induced changes were TTX insensitive, and ouabain-induced changes for inosine, guanosine, 2′-deoxycytidine and 2′-deoxyuridine were TTX sensitive. In contrast, TTX application without depolarisation decreased the extracellular concentrations of hypoxanthine, uridine, inosine, guanosine and adenosine.

Our data suggest that various nucleosides may be released from cells exposed to excessive activity and, thus, support several different lines of research concerning the regulatory roles of nucleosides.

1. Introduction

There are some indications that nucleosides are involved in information transfer between brain cells. ATP has been suggested to be a neurotransmitter (Sperlágh et al., 1995; Sperlágh and Vizi, 1996; Vizi et al., 1997; Zimmermann, 1994), adenosine to be a neuromodulator, modulating for example pain sensation and sleep (Williams, 1990) and protecting against neuronal cell death (Schubert et al., 1997). Uridine is a putative sleep-promoting substance (Honda et al., 1984; Inoue et al., 1995), its plasma concentration changes with the circadian rhythm (el Kouni et al., 1990) and it has hypothermic action (Peters et al., 1987). Other behavioural studies point to the interaction of uridine with the striatal dopaminergic system (Myers et al., 1995). At the cellular level uridine was shown to activate fast transmembrane Ca^{2+} ion fluxes (Kardos et al., 1999). which suggests the possible transmitter function of uridine. Inosine has been suggested to be an endogenous agonist of the benzodiazepine receptors (Costa and Guidotti, 1985). Inosine decreased the rate of discharge in Purkinje cells (Bold et al., 1985) and diminished epileptic seizures independently from benzodiazepine receptor activation (Lapin, 1981). The neurological side-effects of immunosuppressants (Major et al., 1981) anti-tumour agents (Abelson, 1978; Baker et al., 1991; Castellanos and Fields, 1986), and anti-viral agents (Rachlis and Fan-

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ning, 1993), acting on purine and pyrimidine metabolising enzymes, raised the possibility that nucleosides and deoxynucleosides could exert trophic actions on brain cells. Deoxyadenosine induces apoptosis in chick sympathetic neurones (Wakade et al., 1995) and rat chromaffin cells (Wakade et al., 1996) in a DNA synthesis-independent manner (Kulkarni and Wakade, 1996). Deoxycytidine could have a neuroprotective effect independent of DNA synthesis (Wallace and Johnson, 1989), and it probably plays a role in the signal transduction of the nerve growth factor (NGF) (Martin et al., 1990). Guanosine and GTP stimulate proliferation of astrocytes (Rathbone et al., 1992) and increase NGF synthesis and NGF release from cultured astrocytes (Middlemiss et al., 1995). GTP and guanosine enhance neurite outgrowth from pheochromacytoma-derived cell lines (Gysbers and Rathbone, 1996) and from hippocampal neurones (Neary, 1996). The trophic action of guanosine has been suggested to play a part in Parkinson's disease (Loeffler et al., 1998). It has also been proposed that inosine has a trophic effect on neurones (Zurn and Do, 1988) and that inosine mediates the cytoprotective effect of adenosine in astrocyte cultures (Haun et al., 1996). Furthermore, the distribution of nucleosides in cerebrospinal fluid and in brain extracellular space differ, demonstrating that extracellular concentrations of nucleosides are actively regulated in brain (Dobolyi et al., 1998).

As listed above, various nucleosides have significant cell regulatory roles in the nervous system; however, no data are available on the release of nucleosides other than adenosine and its metabolites (White and McDonald, 1990). Using a novel chromatography-based full-scale nucleoside assay (Dobolyi et al., 1998), we investigated the effects of sustained depolarisation induced by high-potassium solution, kainate and ouabain on the brain extracellular nucleoside microenvironment. In addition, the effect of tetrodotoxin (TTX) on basal extracellular concentrations, and TTX sensitivity of kainate- and ouabain-induced release of nucleosides were also studied.

2. Experimental procedures

2.1. Materials

Kainate, ouabain, TTX and the applied chromatographic standards (purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides) were from Sigma Chemical Co. (St Louis, Missouri, USA). Organic solvents and high-performance liquid chromatography (HPLC) eluent components were from Merck Co. (Darmstadt, Germany) and were of HPLC grade.

2.2. Surgery

Experiments were carried out on the basis of local ethical rules in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the European Communities Council Directive 24 November 1986 (86/609/EEC). All efforts were made to minimise animal suffering and to reduce the number of animals. Experiments were performed on male Wistar rats (body weight 350–400 g) under anaesthesia in 0.5–0.8% halothane in air mixture. Rectal temperature was maintained constant with a thermometer-controlled heating lamp. For the implantation of microdialysis probes, two holes of 2.5 mm diameter were drilled bilaterally into the skull above the coordinates of the ventro-posteroletaral and ventro-posteromedial (VPL/VPM) thalamic nuclei (A: −1.9, L: 2.5, V: −7 mm, according to the atlas of Pellegrino and Cushman, 1967).

2.3. Microdialysis

Microdialysis probes were made as described earlier (Juhász et al., 1989). Briefly, a hollow fibre (Travenol, cut-off 5000 D, diameter 0.2 mm, length 3 mm) was adjusted into a 25-gauge stainless steel tubing. Glass capillaries pulled from Jencons glass tubing were used as the inlet and outlet of the probe. These glass capillaries were guided by stainless steel tubes.

The final positions of the probes were attained slowly, in not less than 20 min, in order to reduce tissue damage. Perfusion was performed with artificial cerebrospinal fluid (ACSF), which contained 144 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ in amino acid-free, bidistilled water. The pH of ACSF was adjusted to 7.0 immediately before the beginning of perfusion. The ACSF was tested by HPLC in each experiment. The flow rate was 1 μl/min, and on the basis of our earlier data (Juhász et al., 1989) a 20% concentration recovery was estimated.

Sixty μl samples were collected every 60 min, of which 50 μl was used for nucleoside analysis. The first sample was discarded to allow enough time for the transport barriers to recover after probe penetration caused tissue damage. After two samples had been collected, the perfusion fluid was changed to ACSF containing 0.5–50 μM kainate or 10–100 μM ouabain or high-potassium (27–67 mM NaCl, 80–120 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$), or TTX (1 μM) or kainate (5–50 μM) plus TTX (1 μM) or ouabain (100 μM) plus TTX (1 μM). One probe was used for drug administration, while the contralaterally implanted control probe was perfused with ACSF continuously. Two samples were collected during drug administration, then ACSF was perfused again. Finally, the rats were overdosed with a 4% halothane–air mix-

ture and the positions of the probes were examined on Nissl-stained coronal sections after formaldehyde fixation.

2.4. HPLC technique

A recently developed HPLC method analysing the nucleosides from brain microsamples (Dobolyi et al., 1998) was used in our studies. This method is sensitive and selective enough for the measurement of uracil, hypoxanthine, xanthine, uridine, 2′-deoxycytidine, 2′-deoxyuridine, inosine, guanosine, thymidine and adenosine in microdialysis samples (Fig. 1). Briefly, separation is performed with the Pharmacia LKB SMART chromatographic system on a Hewlett-Packard Hypersyl ODS reversed-phase column (200 × 2.1 mm) filled with 5 μm C18 spherical packing material. Eluent A was 0.02 M formiate buffer, pH = 4.45. Eluent B was 0.02 M formiate buffer containing 40% acetonitrile, pH = 4.45. The gradient profile was as follows: 0% B eluent at 0–2.5 min, 10% at 20 min, 30% at 25 min, and 100% at 38 min. The flow rate was 300 μl/min. Column and detector temperature was 10°C. UV detection was performed at 254 nm. Chromatographic peaks were identified on the basis of the retention times of external standards. Peaks were verified by detection at two wavelengths, and by electrospray tandem mass spectrometry as well (Dobolyi et al., 1998). Chromatograms were evaluated by an interactive batch processing program, written in Matlab for Windows.

2.5. Data analysis

The stable baselines of different substances in the control microdialysis probe were tested with repeated measures ANOVA, or with the Friedmann (nonparametric) ANOVA procedure in case the homogenity and sphericity assumptions were violated (homogenity of variances was tested with Levene's test and sphericity with Mauchly's test). Control values for depolarisations were calculated as averages of the results on the two control samples. Data obtained with perfusion of chemical agents were expressed as percentages of the control values measured in the same microdialysis probe and were compared with Student's *t*-test for correlated samples. Changes were regarded significant if $p < 0.05$. All data are presented as mean ± SEM values. Statistica for Windows 5.0 (StatSoft Inc.) software was used for all statistical analyses.

3. Results

3.1. Extracellular concentrations of nucleosides

The control concentrations calculated from the control values of drug-effect-measuring probes (second-hour and third-hour samples) for 35 animals were as follows: uracil, 1.18 ± 0.21 μM; hypoxanthine, 4.62 ± 0.24 μM; xanthine, 1.26 ± 0.11 μM; uridine, 0.79 ± 0.07 μM; 2′-deoxycytidine, 1.01 ± 0.07 μM; 2′-deoxyuridine, 0.96 ± 0.04 μM; inosine, 0.72 ± 0.06 μM; guanosine, 0.24 ± 0.02 μM; thymidine, 0.22 ± 0.02 μM; and

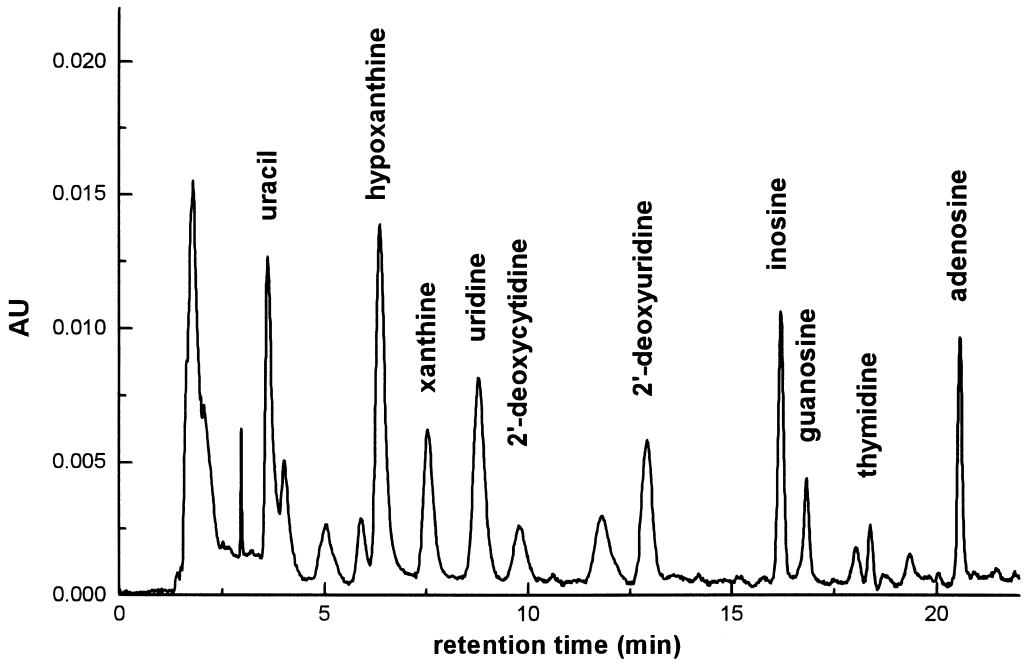

Fig. 1. Chromatogram of 50 μl control microdialysis sample.

adenosine, 0.97 ± 0.04 μM. The nucleoside concentrations in the control probes did not change following the application of chemical agents via the contralateral probes.

3.2. Nucleoside release in response to depolarisation

Kainate, ouabain and potassium were added to the perfusate for 2 h after a 4 h control. There was no significant difference between results obtained in the first and second hours of depolarisation and depolarisation, induced concentrations returned to the baseline values after drug administration was stopped.

When ACSF containing 120 mM K^+ was administered via the microdialysis probe, the extracellular levels of hypoxanthine, uridine, inosine, guanosine and adenosine rose to 201.0 ± 37.8%, 142.8 ± 9.6%, 281.3 ± 88.9%, 334.9 ± 98.8%, and 337.7 ± 45.8%, whereas those of 2′-deoxycytidine and 2′-deoxyuridine decreased to 71.1 ± 10.1% and 60.9 ± 11.2% (Fig. 2), relative to the control values. The extracellular concentration of uracil, xanthine and thymidine did not change significantly. With a lower K^+ concentration (80 mM) in the ACSF, the alterations in the concentrations of adenosine, 2′-deoxycytidine and 2′-deoxyuridine were significant (231.9 ± 45.8%, 68.8 ± 5.0%, and 64.4 ± 2.4% of the control values), and the other compounds changed similarly to those observed at 120 mM K^+.

Perfusion of the glutamate agonist kainate (50 μM) through the microdialysis probe resulted in similar patterns of nucleoside levels (expressed as percentages of the control values): uracil, 207.5 ± 26.8%; hypoxanthine, 321.9 ± 75.1%; xanthine, 422.2 ± 95.7%; uridine, 292.6 ± 72.7%; 2′-deoxycytidine, 95.9 ± 9.3%; 2′-deoxyuridine, 84.7 ± 10.7%; inosine, 230.7 ± 40.6%; guanosine, 292.4 ± 52.6%; thymidine, 129.3 ± 25.2%; and adenosine, 286.9 ± 77.4%. Accordingly, after kainate administration inosine, guanosine and adenosine levels changed similarly as after the administration of ACSF containing 120 mM K^+, while hypoxanthine and uridine levels rose by an even greater extent (Fig. 2). Further considerable differences between the effects of the two types of depolarisations were that the xanthine concentration was elevated, while those of 2′-deoxyuridine and 2′-deoxycytidine were not decreased by kainate (Fig. 2).

Extracellular levels of nucleosides — after 100 μM ouabain (Fig. 2) was administered — were as follows (expressed as percentages of the control values): uracil, 98.7 ± 10.7%; hypoxanthine, 187.4 ± 27.5%; xanthine, 161.5 ± 23.0%; uridine, 136.1 ± 7.5%;

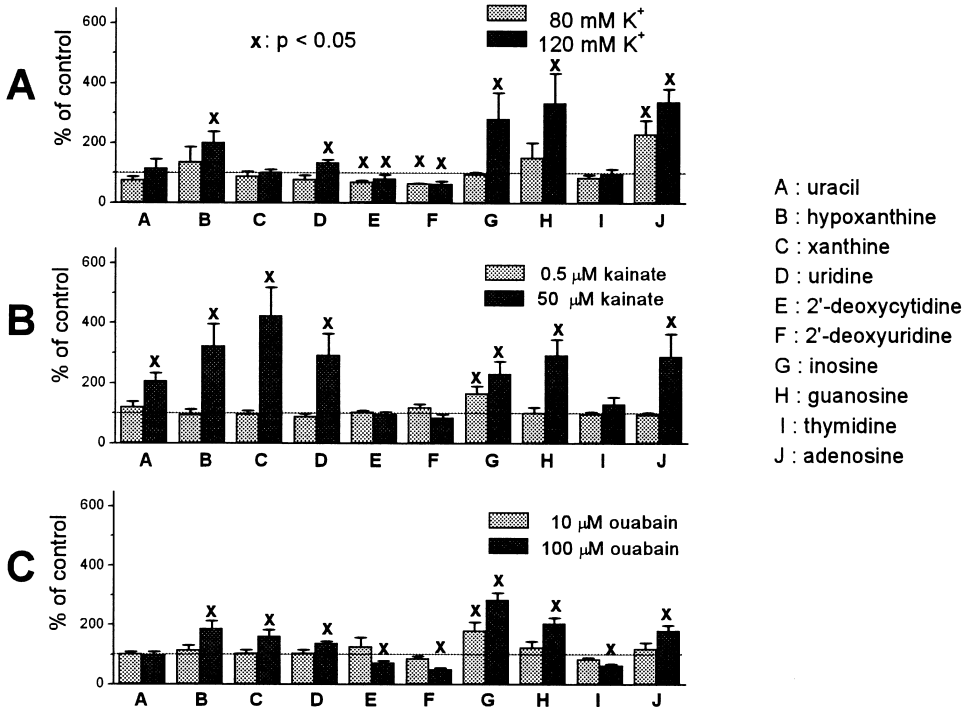

Fig. 2. Extracellular concentration changes of nucleosides in percentage of the control under (A) high potassium-, (B) kainate-, and (C) ouabain-induced depolarisation. Two different concentrations were used for each drug to show concentration dependent changes. The horizontal line is at 100% representing the control value. Crosses represent significant changes in nucleoside concentrations in response to high potassium, kainate, or ouabain perfusion as compared to the control nucleoside concentrations.

2′-deoxycytidine, 72.8 ± 7.3%; 2′-deoxyuridine, 50.8 ± 5.3%; inosine, 283.8 ± 26.0%; guanosine, 203.3 ± 21.4%; thymidine, 82.9 ± 15.9%; and adenosine, 181.2 ± 18.0%. The application of 10 μM ouabain resulted in a significant change only in the extracellular level of inosine (180.2 ± 29.4%).

Tissue damage surrounding the microdialysis probes did not increase after the administration of the depolarising agents, according to Nissl-stained coronal sections of the formaldehyde fixated brains.

3.3. Effects of TTX on nucleoside concentrations

The perfusion of 1 μM TTX through the microdialysis probe decreased the extracellular concentrations of hypoxanthine, uridine, inosine, guanosine and adenosine to 59.5 ± 10.0%, 65.1 ± 9.8%, 63.5 ± 12.9%, 65.4 ± 11.7% and 52.1 ± 14.9% of the control values, respectively, whereas the extracellular levels of the other measured compounds did not change (Fig. 3).

Concentration changes in response to kainate (50 and 5 μM) administration were TTX insensitive (Fig. 4). In case of ouabain (100 μM) induced depolarisation, TTX (1 μM) decreased the degree of changes for 2′-deoxycytidine, 2′-deoxyuridine, inosine and guanosine from 72.8 ± 7.3% to 102.3 ± 11.1%, from 50.8 ± 5.3% to 76.0 ± 10.2%, from 283.8 ± 26.0% to 121.6 ± 12.2% and from 203.3 ± 21.4% to 101.1 ± 12.6% (expressed as percentages of the control values), respectively (Fig. 4). In addition, elevated hypoxanthine, uridine and adenosine levels tended to decrease (Fig. 4).

4. Discussion

4.1. Measuring cellular microenvironment with microdialysis

In steady state, microdialysis reflects the balance of release and uptake of compounds in the extracellular space. However, the penetration of a microdialysis probe causes a temporary disturbance by damaging the brain tissue and blood-brain barrier, but it is widely accepted that extracellular concentrations of biomolecules are re-established rapidly (Benveniste and Huttemeier, 1990; Gratzl et al., 1991). We found steady concentrations of nucleosides from the second hour after the probes were introduced. This made the investigation of depolarisation-induced changes in the nucleoside microenvironment in the central nervous system possible after 2 h recovery.

Time resolution of microdialysis method is in the minute range (Juhász et al., 1989). The large injection volume necessary for our recently developed full-scale nucleoside assay (Dobolyi et al., 1998) was the reason of 60 min sampling time in our studies. In turn, we measured a long term equilibrium concentration change in nucleosides during sustained depolarisation paradigms. We were not able to achieve better time resolution because microdialysis method makes a diluted sample from the extracellularly available nucleosides, approaching the detection limits of HPLC for several nucleosides including uridine, guanosine, 2′-deoxyuridine, 2′-deoxycytidine and thymidine (Dobolyi et al., 1998).

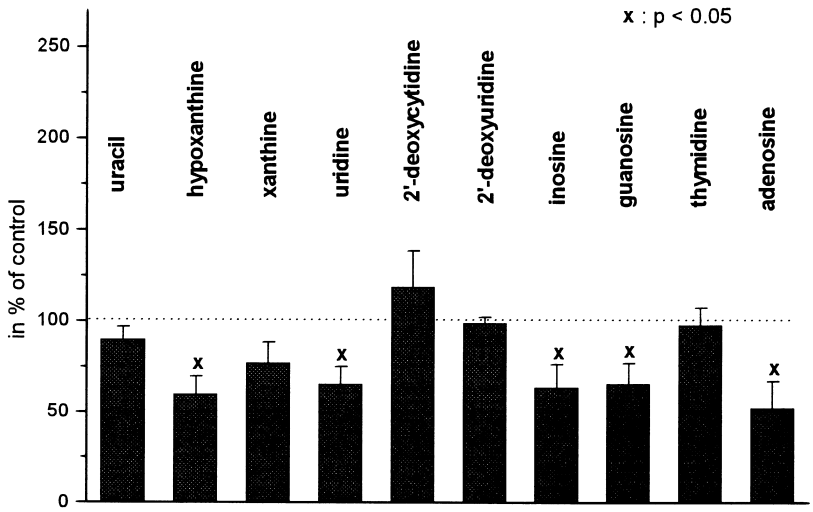

Fig. 3. Effects of 1 μM TTX perfusion on basal extracellular nucleoside concentrations. Data are expressed as percentages of the control levels. The control levels are calculated as the averages of the results for the samples, 2 and 3 h after implantation of the probe. Values are averages ± SEM of data on four animals. Horizontal linea are at 100% representing the control value.

4.2. Depolarisation paradigms and their limitations

Administration of depolarising agents in the perfusion solution induces sustained depolarisation of the surrounding tissue as they diffuse into the extracellular space. Diffusion of compounds from the dialysis probe is slow and the actual concentration in the tissue cannot be estimated properly because of unmeasurable in vivo transport and uncertain size of the exposed tissue. We estimated a mass transport efficiency of 20% on the basis of in vitro model studies (Juhász et al., 1989).

Depolarisation by high-potassium solution and kainate may be highly energy-consuming for the cells because the cells strive to repolarise their plasma membrane by speeding up the active transport (Erecinska and Silver, 1989). The negative energy balance itself could induce changes in the release of nucleosides. However, ouabain can depolarise cells by blocking the Na^+/K^+ ATPase thus, a micromolar range concentration of ouabain (the approximate extracellular concentration in our studies) has little or no effect on the energy balance (Senatorov et al., 1997). Our findings on ouabain application (Fig. 2) suggest that the depolarisation-induced changes in extracellular nucleoside concentrations are at least partly a direct consequence of membrane depolarisation.

There are indications that swelling induced by sustained depolarisation could make the neurones die. The applied drug concentrations and our histological data do not suggest the occurrence of cell death in our experiments. It cannot be excluded, however, that the measured changes in the nucleoside concentrations can be related to compensatory volume regulation.

4.3. Changes in neuronal nucleoside microenvironment

Our results relating to the release of adenosine, inosine and hypoxanthine in thalamic VPL/VPM nuclei supplement data reported in other brain regions. Following several reports on adenosine release in response to various types of depolarisation in vitro (White and McDonald, 1990), K^+-evoked depolarisation (Pazzagli et al., 1993) and kainate-evoked depolarisation (Carswell et al., 1997) were also applied in microdialysis experiments. It was demonstrated in vivo that such procedures result in adenosine release in the rat striatum and hippocampus, respectively. TTX-dependence of the extracellular adenosine level was described similarly by both reports. Inosine and hypoxanthine have been reported to be released from the rat striatum following electrical and kainate-evoked stimulation (Sciotti et al., 1993). The experiments described in the present paper show that all these findings on adenosine, inosine and hypoxanthine can be reproduced in the thalamus, and the data also demonstrated the release of these compounds when ouabain was perfused via the microdialysis probes. Since hypoxanthine, inosine and xanthine can be formed from adenosine

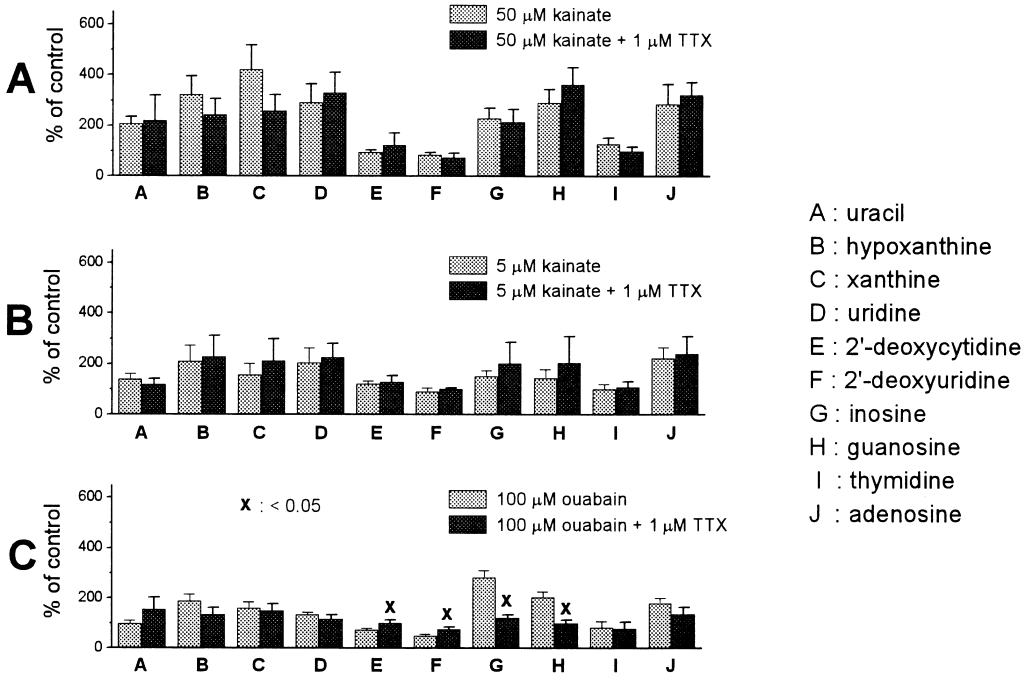

Fig. 4. Effects of 1 μM TTX on extracellular concentration changes of nucleosides evoked by (A) high concentration kainate, (B) low concentration kainate, and (C) ouabain perfusion. Data obtained in the absence and in the presence of TTX are both expressed in percentage of the control values. Control values were measured by the perfusion of ACSF without any drug. Crosses represent significant reversal effects by TTX co-application as compared to drug-evoked changes of nucleoside concentrations in the absence of TTX.

(Lloyd and Fredholm, 1995), the increases in their levels could be secondary. In addition to the increases in the concentrations of adenosine and metabolically related compounds, our experiments provided the first evidence of changes in the extracellular levels of other nucleoside compounds, i.e. guanosine, uridine, 2′-deoxyuridine and 2′-deoxycytidine, in different depolarisation paradigms. The changes in these concentrations are particularly noteworthy because metabolic conversion from adenosine to guanosine, uridine, 2′-deoxyuridine or 2′-deoxycytidine is not possible.

When considering the mechanisms of changes in the extracellular concentrations of nucleosides, there are several possibilities including depolarisation- or spike-induced releases, reduced uptake capacities, extracellular formation from nucleotides or other nucleosides, rearrangements in metabolic processes of cells or any of the combination of these for each particular substance. To address the involvement of spike activity in the changes of extracellular nucleoside concentrations, the TTX dependency of the kainate- and ouabain-induced changes were examined. These experiments revealed some differences in the mechanisms of alterations of the extracellular nucleoside concentrations by these two drugs. Changes in spike activity by depolarisation seem not to be involved for the effects of kainate because the concentration changes were not at all reversed by the co-application of TTX. This finding is in contrast to that found in the rat hippocampus (Carswell et al., 1997), suggesting that presynaptic kainate receptors (Malva et al., 1998) may be predominant in the thalamus and directly trigger adenosine release. In contrast to kainate, the effects of ouabain were found to be partially TTX-sensitive which supports some role of altered spike activity in the changes of the extracellular concentrations of some nucleosides, especially for those where the effect of ouabain was almost completely reversed (2′-deoxycytidine, 2′-deoxyuridine, inosine and guanosine) by the co-application with TTX. Another possible explanation for the partial TTX sensitivity of ouabain-induced release is that TTX could decrease the degree of ouabain-induced depolarisation. Thus, a stronger evidence for the possible involvement of spike activity in the changes of extracellular hypoxanthine, uridine, inosine, guanosine and adenosine concentrations is the decrease in their basal extracellular levels by perfusing TTX without depolarising agents. To address the involvement of nucleoside transporters and extracellular formation from nucleotides, we tried to apply the nucleoside uptake blocker dipyridamole and the ecto-5′-nucleotidase blocker α,β-methylene ADP, respectively. We failed, however, because these compounds are structurally related to nucleosides; they formed an enormous peak in the chromatogram of our method, masking many nucleosides. In addition, they bound to our chromatographic column irreversibly when applied in appropriate concentrations.

4.4. Possible physiological role of extracellular nucleosides

Adenosine is a well-established neuromodulator (Williams, 1990) and non-synaptic interaction between neurones has been suggested to play a part in brain function (Vizi, 1984). Although other nucleosides have not been suggested to play a part in neurotransmission, various data suggest that some of them could be neuroactive compounds: guanosine, which has trophic effects on neurones and glial cells (Gysbers and Rathbone, 1996; Loeffler et al., 1998; Middlemiss et al., 1995, Neary, 1996; Rathbone et al., 1992); inosine, which is suggested to be a cytoprotective agent (Haun et al., 1996) and also a trophic agent (Zurn and Do, 1988); uridine, which is assumed to be a sleep-promoting substance (el Kouni et al., 1990; Honda et al., 1984; Inoue et al., 1995) and was suggested to be a neurotransmitter on the basis of its effects on fast transmembrane Ca^{2+} ion fluxes in synaptosomes (Kardos et al., 1999); and deoxynucleosides, which may modulate cell survival (Kulkarni and Wakade, 1996; Martin et al., 1990; Wakade et al., 1995; Wakade et al., 1996; Wallace and Johnson, 1989).

In conclusion, our results demonstrate that depolarisation can significantly change the extracellular concentrations of many nucleosides. Although at the current state of knowledge on non-adenosine nucleosides, it would be purely speculative to suggest any particular physiological role of depolarisation-released nucleosides in general, our findings strengthen several different lines of research concerning the regulatory roles of various nucleosides.

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