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Uridine activates fast transmembrane Ca^{2+} ion fluxes in rat brain homogenates

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THE excitatory actions of the pyrimidine nucleoside uridine, and the nucleotides UDP and UTP, as well as the purine nucleotide ATP, were studied by fluorescent labeling of Ca^{2+} and K^+ ion fluxes on the time scale of 0.04 ms to 10 s in resealed plasmalemma fragments and nerve endings from the rat cerebral cortex. Two phases of Ca^{2+} ion influx with onsets of a few milliseconds and a few hundred milliseconds, showing different concentration dependencies, agonist sequences and subcellular localizations were distinguishable. [^{3}H]Uridine identified high (K_D ~15 nM) and low affinity (K_D ~1 μM) specific binding sites in purified synaptosomal membranes. Labeled uridine taken up by synaptosomes in a dipyridamole-sensitive process was released by depolarization (1 mM 4-aminopyridine). Taken together, these results may qualify uridine as a neurotransmitter. *NeuroReport* 10:1577–1582 © 1999 Lippincott Williams & Wilkins.

Key words: Binding; Ca^{2+} influx; Rat cerebral cortex; Release; Uptake; Uridine

Introduction

Excitatory receptors responsive to extracellular pyrimidine nucleotides have been identified [1–6]. Although there is evidence for uridine acting as a CNS depressant [7,8], suggestions for an excitatory role in the CNS have so far not been supported by any direct evidence at the molecular level [9]. Hepatic uridine [10] enters the extracellular space of the brain, and subsequently brain cells, from the blood, where it can be phosphorylated and incorporated into RNA or be catabolized to uracil [11,12]. Since the extracellular concentration of uridine has been found to be increased by high K^+ in the rat thalamus [13], it was of interest to study uridine-responsive cation fluxes through brain membranes. Here we describe a fluorescent tracer method to study uridine-activated Ca^{2+} and K^+ ion translocation in suspensions of resealed plasmalemma fragments and nerve endings on the time scale achievable by stopped-flow spectroscopy. Uptake, release and binding of radiolabeled uridine in suspensions of synaptosomes and synaptosomal membranes, respectively, were also measured. Cerebral–cortical suspensions were controlled by HPLC analyses of endogenous purines and pyrimidines.

Materials and Methods

Male Wistar rats (4–6 weeks old) were from LATI (Gödölló, Hungary). [^{14}C]Uridine (520 mCi/mmol) and [^{3}H]uridine (40 Ci/mmol) were from Amersham. The membrane impermeant forms of fluorescent Ca^{2+} and K^+ ion indicators, Bisfura2 and PBFI, respectively, were purchased from Molecular Probes. Uridine, aprotinin, leupeptin, antipain, pepstatin A, butylated hydroxy-toluene (BHT), phenylmethanesulfonyl fluoride (PMSF) and 4-aminopyridine (4-AP) were from Sigma. Dilazep, dipyridamole and ouabain were from Tocris, RBI and Fluka, respectively. HEPES was obtained from Calbiochem. DMSO, Acetonitrile, $CaCl_2$ and $MgCl_2$ were from Merck. Other chemicals were from Reanal. Buffers with and without 1.3 mM $CaCl_2$ added (A and B, respectively) had the following composition: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES adjusted to pH 7.4 with NaOH. Buffer C was 5 mM TRIS adjusted to pH 7.4 at 4°C with HCl. Buffer D had the following composition: 0.32 M sucrose, 1 mM PMSF, 0.01 mg/ml aprotinin, leupeptin, antipain, pepstatin A, 0.005 mg/ml each, 0.02 mM BHT, 10 mM HEPES adjusted to pH 7.4 with NaOH. Buffer E com-

prosed 1 mM EGTA-TRIS, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES adjusted to pH 7.4 with NaOH.

Purified synaptosomal fraction (SYN) was prepared from cerebral cortices of rats as described previously [14]. Rats were decapitated with a guillotine. Cortices were dissected and homogenized (~5–6 g) in 10 vol. iced 0.32 M sucrose solution with a Potter–Elvehjem homogenizer at 1400 r.p.m. The homogenate was pelleted at $1500 \times g$ for 10 min at 4°C and the supernatant was further centrifuged at $9500 \times g$ for 20 min at 4°C. The pellet was resuspended in iced 0.32 M sucrose solution and layered on the top of 0.8 M iced sucrose solution and centrifuged at $9500 \times g$ for 25 min at 4°C. Combined 0.80 M sucrose fractions were gradually diluted with buffer A to 0.40 M and centrifuged at $20\,000 \times g$ for 15 min at 4°C. The resulting pellet was suspended in 1.35 ml buffer A to give a protein concentration of 19–20 mg/ml. Protein was measured by the Folin reagent method [15]. The SYN kept on ice for 2 h (aged SYN) was used in [^{14}C]uridine uptake and [^{3}H]uridine release assays or was frozen at −80°C for preparation of synaptosomal membrane fraction. In the transmembrane Ca^{2+} ion flux measurements, the SYN pellets kept on ice were suspended in buffer B to give a protein concentration of 0.3 mg/ml, preincubated at 30°C for 5 min and used immediately (fresh SYN).

Native plasma membrane vesicle fraction (NPMV) from the cerebral cortex of rat was prepared as described previously [16]. The cortex was dissected and homogenized in 30 ml buffer D. Thereafter 30 ml buffer A was added and the suspension was centrifuged at $270 \times g$ for 4 min at 4°C. The supernatant was centrifuged at $6500 \times g$ for 20 min at 4°C followed by resuspension of the pellet in a Potter–Elvehjem glass–Teflon homogenizer by hand. Thereafter, aliquots of the suspension were centrifuged at $3500 \times g$ for 15 min at 4°C. The resulting pellets kept on ice were resuspended as above in buffer B to give a protein concentration of 0.3 mg/ml, preincubated at 30°C for 5 min and used immediately in transmembrane Ca^{2+} ion flux measurements (fresh NPMV).

For [^{3}H]uridine binding measurements, synaptosomal membrane fraction (SYNM) was prepared from SYN kept at −80°C for several weeks. An aliquot (corresponding to 5.0–5.5 g original tissue in 1.35 ml buffer A) was thawed, homogenized in 30 ml buffer C with a Potter–Elvehjem glass–Teflon homogenizer at 1400 r.p.m. and kept at 4°C for 2 h. The homogenate was then centrifuged at $20\,000 \times g$ for 20 min at 4°C. The pellet was rehomogenized as above and centrifuged at $20\,000 \times g$ for 20 min at 4°C twice. For HPLC analyses of purine and pyrimidine derivatives, the pellet was resuspended in 1.35 ml buffer C and centrifuged at $18\,865 \times g$ for 15 min at 4°C. The resulting supernatant was recentrifuged at $18\,865 \times g$ for 15 min at 4°C (first HPLC sample), whereas the pellet was frozen at −80°C for 16 h. The thawed pellet was resuspended in 30 ml buffer C and pelleted at $20\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in 1.35 ml buffer C and centrifuged at $18\,865 \times g$ for 15 min at 4°C. The resulting supernatant was recentrifuged at $18\,865 \times g$ for 15 min at 4°C (second HPLC sample) and the pellet was frozen as above. This thawing–washing–sampling–freezing cycle was repeated once again with divided pelleting (3×0.45 ml) to give the third HPLC sample. Thereafter the three thawed pellets were homogenized in buffer C (10 ml each) and centrifuged at $20\,000 \times g$ for 20 min at 4°C twice. Thereafter, two pellets were resuspended in buffer A to give a protein concentration of 0.4–0.5 mg/ml and used in [^{3}H]uridine binding experiments. One pellet was resuspended in 0.45 ml buffer C and centrifuged at $18\,865 \times g$ for 15 min at 4°C. The resulting supernatant was recentrifuged at $18\,865 \times g$ for 15 min at 4°C to give the fourth HPLC sample. By taking into account an ~40-fold dilution of SYNM (cf. matching sample 4 in Table 1) the concentrations of

Table 1. Concentrations (in μM) of hypoxanthine (HX), xanthine (X), uridine (U), inosine (I), guanosine (G) and adenosine (A) in samples of fresh and aged suspensions of SYN, NPMV and SYNM purification procedure as determined by HPLC analysis

Sample	[HX]	[X]	[U]	[I]	[G]	[A]
SYN fresh	0.45	0.11	0.29	1.53	0.34	1.95
SYN aged	1.63	0.39	0.73	4.96	0.68	3.74
NPMV fresh	0.17	0.05	0.11	0.28	0.09	0.62
NPMV aged	0.96	0.53	0.65	2.76	0.46	1.31
SYNM 1.	5.32	4.43	0.31	5.04	0.01	0.44
SYNM 2.	0.23	0.46	0.04	0.33	0.11	0.84
SYNM 3.	0.06	0.20	0.04	0.07	0.06	0.23
SYNM 4.	0.03	0.03	0.02	0.01	0.07	0.09
Buffer A	0.03	0.02	0.01	0.01	0.01	0.01

endogenous purines and pyrimidines are less (< 1 nM) than their concentrations in the buffer in the [^{3}H]uridine binding experiments.

To determine purines and pyrimidines in samples from rat brain homogenates, analysis of 100 µl samples was performed in a Pharmacia-LKB SMART HPLC/UV detection system as described recently [13]. For separation, a Hewlett-Packard Hypersyl ODS reversed phase column was used in combination with the following eluents: 0.02 M formate buffer pH 4.45 (A) and 0.02 M formate buffer pH 4.45 containing 40% acetonitrile (B). The gradient profile was 0% eluent B in 0–10 min, 10% eluent B at 22 min, and 100% eluent B at 30 min with a flow rate of 3.5 ml/min. u.v. absorption was monitored at 254 nm. The column and detector temperature was 15°C. Chromatographic peaks from samples were identified on the basis of the retention times of known standards. Sampling from different types of rat brain homogenates were repeated three times. Data are expressed as mean ± 30% (s.e.) (Table 1).

[^{14}C]Uridine uptake by SYN was measured as described previously [17]. Aliquots of SYN (50 µl) were preincubated with 50 µl buffer A with or without the test compounds (dipyridamole, dilazep and ouabain) for 2 min. A 10 µl aliquot of the above suspension and 20 µ [^{14}C]uridine solution were placed separately on the opposite side of a plastic tube and preincubated at 37°C for 2 min. The assay medium contained 10 µM (5 µCi/ml) [^{14}C]uridine and 0.25% DMSO after vortex mixing. After 1 min incubation at 37°C (or at 4°C), the uptake was terminated by the addition of 0.9 ml iced buffer A containing 50 µM dipyridamole (stop solution). The suspension was immediately filtered through glass fiber filters (Whatman GF/B), and washed (2 × 4 s) with 10 ml iced stop solution. The radioactivity of samples was counted in HiSafe II (LKB) scintillation mixture (efficiency of ~80%).

[^{3}H]Uridine release by SYN was measured by the method described previously [18] with slight modifications. An aliquot of SYN (200 µl) was prewarmed at 37°C for 2 min. Loading was initiated by the addition of prewarmed buffer A (200 µl) containing [^{3}H]uridine (final concentration 5 µM, 200 µCi/ml). After 1 min incubation at 37°C the suspension was rapidly filtered through a glass fiber filter (Whatman GF/B) and washed (2 × 4 s) with 10 ml prewarmed (37°C) buffer A or E. Buffer A or E (800 µl) was layered on top of SYN loaded with [^{3}H]uridine. After 10 s the solution was rapidly filtered and replaced with 800 µl fresh buffer A or E. In a typical experiment, the radioactivity of 800 µl/10 s fractions (expressed as a percentage of the actual [^{3}H]uridine content of SYN), obtained from consecutive application of buffer A or E (1–5), 1 mM 4-AP in buffer A or E (6–8) and buffer A or E (9,10) was measured in HiSafe II (LKB) scintillation mixture (efficiency of ~25%). [^{14}C]Uridine uptake and [^{3}H]uridine release experiments were carried out in duplicate and were repeated three times. Data are expressed as mean ± s.e. and were analyzed using one-way analysis of variance (ANOVA, ORIGIN ver 3.5) for *post hoc* comparisons. A value of $p < 0.05$ was considered significant.

For [^{3}H]uridine binding, aliquots (450 µl) of the SYNM were incubated with equal amounts of [^{3}H]uridine (final concentration: 1–300 nM, 0.04–13.4 µCi/ml) in the absence or (to define non-specific binding) the presence of 100 µM unlabeled uridine in buffer A, for 1 h at 5°C. Samples of 400 µl suspension were then centrifuged in Eppendorf tubes at 18 865 × *g* for 3 min at 5°C, the supernatants were vacuum aspirated and the pellets were immediately washed (2 × 4 s) with 3 ml iced buffer A. The resulting pellets were solubilized in 10% SDS solution (100 µl each), transferred into scintillation vials and combined with the 50 µl 10% SDS solution used to rinse the Eppendorf tubes. The radioactivity of samples was counted in HiSafe II (LKB) scintillation mixture (efficiency of ~25%). [^{3}H]Uridine binding experiments were carried out in duplicate and were repeated three times. The equilibrium [^{3}H]uridine binding parameters were determined by fitting the equilibrium binding data measured with different [^{3}H]uridine concentrations, corrected for the presence of unlabeled uridine in the buffer, to the Cooperative Binding Isotherm model of MicroMath Scientist Microsoft Windows version 2.0.

Transmembrane Ca^{2+} ion and K^+ ion fluxes in suspensions of NPMV were followed by rapid mixing of NPMV or SYN in 75 µl buffer (0.3 mg protein/ml, ~3% vol/vol) with an equal volume of buffer solution containing the membrane-impermeant form of the fluoprobe, in the presence or absence of U, UDP, UTP and ATP at various concentrations. Under these conditions, the decrease of fluorescence intensity of the Ca^{2+} or K^+ ion fluoprobes, monitored at 500 nm using a 260–390 nm excitation filter, corresponded to the influx of Ca^{2+} or K^+ ion into NPMV or SYN. Fast mixing of thermostatted reactants (30.0 ± 0.1°C) was performed in a SF-61 DX2 stopped-flow uv/fluorescence detection system (HiTech) supported by the Microsoft Windows 95 KinetAssyst computer program (HiTech) [16]. The percentage fluorescence intensity over time was recorded on a logarithmic time base. Traces comprise 640 data sets acquired automatically. Transformation of the percent fluorescence data into the percentage change of fluores-

cence was performed with subtraction of background trace measured without additives. Transmembrane Ca^{2+} and K^+ ion flux measurements were performed in buffer B and A, respectively. Concentrations of the fluoprobes were 0.5 μM (Bisfura2) and 10 μM (PBFI). The equilibrium dissociation constants of Bisfura2-Ca^{2+} and PBFI-K^+ complexes are 525 nM and 44 mM, respectively [19]. Matching the calculated F_{max} values, the maximal percentage change of fluorescence observed in Ca^{2+} and K^+ ion flux measurements was 5% (Ca^{2+} flux) and 2% (K^+ flux). Transmembrane Ca^{2+} ion and K^+ ion flux experiments were carried out in quintuplicate and were repeated at least three times. Data are plotted so that the positive values represent the influx of Ca^{2+} ion and K^+ ion. The kinetic rate parameters for Ca^{2+} ion flux were determined by fitting (MicroMath Scientist Microsoft Windows version 2.0) the relative change of fluorescence (F/F_{max}) against time, measured with different concentrations, to the equation [20]: $F/F_{max} = 1-\exp\{-[J_A(1-\exp-\alpha t)/\alpha] + J_B[(1-\exp-\beta t)/\beta]\}$, where F/F_{max} is the fraction of Ca^{2+} ion flux that has occurred in time t, J_A and J_B are the initial values of the first-order rate constants for the Ca^{2+} ion flux mediated by receptors A and B. Alpha and beta are first-order rate constants for desensitization of the activities in the fast and slow phases.

Results

Specific [^{3}H]uridine binding was identified in synaptosomal membranes from the rat cerebral cortex at 5°C. In saturation studies the radioligand showed high and low affinity binding sites: $K_D(high) = 14.5 \pm 3.6$ nM, $B_{max}(high) = 900 \pm 400$ fmol/mg protein, $K_D(low) = 0.96 \pm 0.30$ μM, $B_{max}(low) = 14.2 \pm 2.5$ pmol/mg protein. [^{14}C]Uridine uptake in rat cerebral–cortical synaptosomes was 10.8 ± 1.8 pmol/mg protein/min at 37°C and 2.4 ± 1.0 pmol/mg protein/min at 4°C. [^{14}C]Uridine uptake at 37°C was significantly ($p < 0.01$) inhibited by 25 μM dipyridamole and by 25 μM dilazep (3.5 ± 0.6 pmol/mg protein/min and 2.5 ± 0.4 pmol/mg protein/min, respectively), but was insensitive to 1 mM ouabain (11.1 ± 1.9 pmol/mg protein/min). The rate of [^{3}H]uridine release in rat cerebral–cortical synaptosomes was monitored at 37°C under different conditions, either with Ca^{2+} ion (buffer A) or without Ca^{2+} ion (buffer E) in the presence or absence (basal) of depolarizing 4-AP (1 mM). Rates of basal and depolarization-induced [^{3}H]uridine release were invariably 0.55 ± 0.05%/s and 0.75 ± 0.05%/s, respectively, when Ca^{2+} ion was present or absent. The value of 36 ± 9% ($p < 0.05$) over basal fits *in vivo* data for uridine release (~40%) [13] as well as data on high-[K^+]-induced Ca^{2+}-independent [^{3}H]adenosine release in cultured cerebellar granule cells ($\sim 50 \pm 5$%) [21]. By comparison, the high-[K^+]-induced adenosine release was ~180% *in vivo* [13]. It is noteworthy that ~70% of [^{3}H]adenosine released from brain synaptosomes was uridine-sensitive [22].

Uridine activated concentration-dependent Ca^{2+} ion influx in NPMV (Fig. 1A). As the concentration of uridine increased so the rate of influx and desensitization of the activities also increased. At 30 nM uridine the influx showed a single slowly desensitizing phase, but at 300 nM or above two phases were observed (Fig. 1A,B). Influx in SYN evoked by 30 μM uridine was best fitted with a single, slowly-desensitizing phase (Fig. 1B). UDP (3 μM) evoked similar fast-phase and slow-phase inward Ca^{2+} fluxes. ATP (3 μM) activated a major fast-phase, whereas UTP (3 μM) activated a major slow-phase influx (Fig. 1C). UTP (3 μM) activated a fast K^+ ion influx. There was no influx of K^+ ion in the presence of 3 μM uridine (Fig. 1D).

Discussion

Here we report the transmembrane Ca^{2+} ion influx evoked by uridine in rat cerebral–cortical homogenates. The fast-phase (onset of a few milliseconds) of the fluxes showed a U = ATP > UDP > UTP agonist sequence whereas the slow-phase (onset of a hundred milliseconds) obeyed the UTP ≥ U ≥ UDP > ATP agonist sequence. The two kinetically distinguishable activities showed different subcellular localization as slow-phase influx evoked by uridine being more abundant in nerve endings. Fast inward K^+ ion influx was more affected by UTP than by ATP, while uridine was inactive. Taken together, these results suggest multiplicity of uridine signalling via different Ca^{2+}-permeable nucleotide receptors or coupling to a known Ca^{2+} channel. The slow- and fast-phase Ca^{2+} influx seen with uridine may be mediated by the high and low affinity uridine binding sites, respectively. It is conceivable that the dipyridamole-sensitive uridine uptake is a part of the nucleoside transport system and as such, a potential source of uridine. The Ca^{2+}-independent, depolarization-evoked release of uridine through the plasma membrane corroborates previous suggestions [23] for the existence of a non-vesicular release mechanism.

Conclusions

In addition to the presence of specific uridine binding sites, facilitated transmembrane influx of uridine and depolarization-induced uridine release,

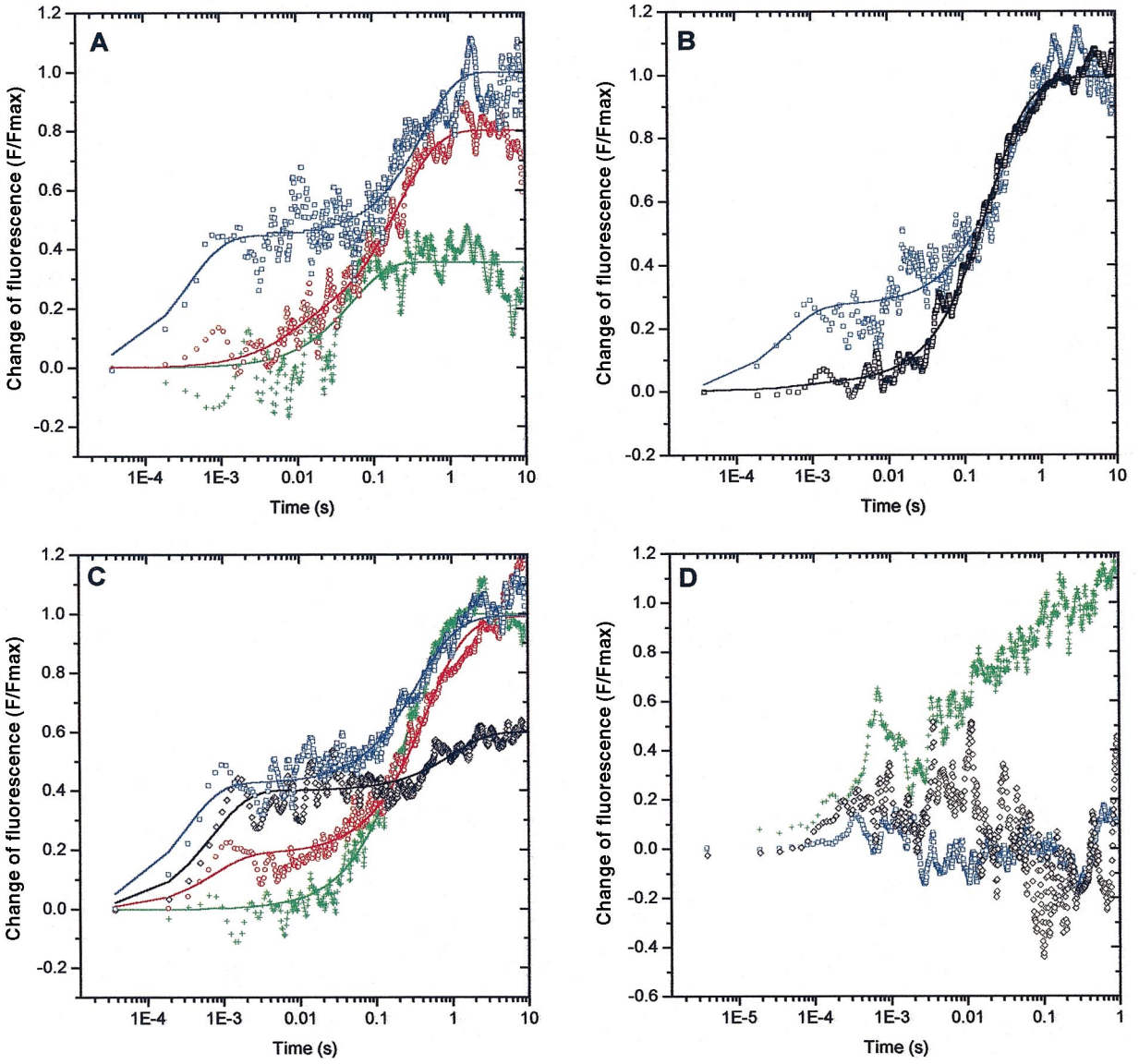

Fig 1. Effects of uridine, UDP, UTP and ATP on the rates of transmembrane Ca^{2+} and K^+ ion fluxes in suspensions of resealed plasmalemma fragments (NPMV) and nerve endings (SYN) from the rat cerebral cortex at 30°C. (**A**) Uridine-activated transmembrane Ca^{2+} ion influx and desensitization of the activities in suspensions of NPMV. Symbols: 0.03 μM uridine, green +; 0.3 μM uridine, red circle; 3 μM uridine, blue square. The lines are computed by using the equation given above for $J_A = 1.6 \pm 0.1$/s, $\alpha = 17 \pm 66$/s, $J_B = 5.2 \pm 0.7$/s, $\beta = 15 \pm 14$/s ([U] = 0.03 μM, green); $J_A = 18.1 \pm 2.4$/s, $\alpha = 114 \pm 20$/s, $J_B = 3.7 \pm 0.1$/s, $\beta = 2.5 \pm 0.1$ ([U] = 0.3 μM, red); $J_A = 1470 \pm 470$/s, $\alpha = 2950 \pm 940$/s, $J_B = 4.6 \pm 0.1$/s, $\beta = 2.1 \pm 0.1$ ([U] = 3 μM, blue). (**B**) Comparison of uridine-activated Ca^{2+} ion translocation in suspensions of NPMV (blue squares) and SYN (black squares). The lines are computed using the equation in the text for $J_A = 960 \pm 820$/s, $\alpha = 4130 \pm 3560$/s, $J_B = 5.6 \pm 0.1$/s, $\beta = 1.7 \pm 0.1$ (NPMV, [U] = 30 μM, blue); $J_A = 41 \pm 63$/s, $\alpha = 1670 \pm 2572$/s, $J_B = 4.5 \pm 0.1$/s, $\beta = 0.9 \pm 0.1$ (SYN, [U] = 30 μM, black). (**C**) Comparison of Ca^{2+} ion translocations activated by uridine, UDP, UTP and ATP. Symbols: 3 μM uridine, blue squares; 3 μM UDP, red circles; 3 μM UTP, green +; 3 μM ATP, black diamonds. The lines are computed using the equation in the text for $J_A = 1420 \pm 240$/s, $\alpha = 2570 \pm 440$/s, $J_B = 2.7 \pm 0.1$/s, $\beta = 0.6 \pm 0.1$ ([U] = 3 μM, blue); $J_A = 250 \pm 7$/s, $\alpha = 1220 \pm 40$/s, $J_B = 2.1 \pm 0.1$/s, $\beta = 0.5 \pm 0.1$ ([UDP] = 3 μM, red); $J_A = 0.6 \pm 0.2$/s, $\alpha = 10 \pm 9$/s, $J_B = 3.2 \pm 0.1$/s, $\beta \sim 0$ ([UTP] = 3 μM, green); $J_A = 560 \pm 60$/s, $\alpha = 1090 \pm 110$/s, $J_B = 0.30 \pm 0.01$/s, $\beta = 0.75 \pm 0.02$ ([ATP] = 3 μM, black); (**D**) Comparison of K^+ ion translocation activated by uridine, UTP and ATP. Symbols: 3 μM UTP, green +; 3 μM ATP, black diamonds; 3 μM uridine, blue squares.

our data provide evidence that uridine might be released to act as a fast excitatory transmitter in the rat cerebral cortex.

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