P2X Receptor Activation Elicits Transporter-Mediated Noradrenaline Release from Rat Hippocampal Slices

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ABSTRACT

This study was designed to test the hypothesis of whether activation of presynaptic P2X receptor-gated ion channels elicits noradrenaline release from central catecholaminergic terminals. ATP, α,β-methylene-adenosine 5'-triphosphate (αβME-ATP), and ADP elicited concentration-dependent [3H]noradrenaline outflow from superfused rat hippocampal slices with the following rank order of agonist potency: αβM-ATP > ATP > ADP. Among P2 receptor antagonists, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (30 μM), 4',4',4',4'-[carbonylbis(mimo-5,1,3-benzenetriyl-bis(carbonylimino)]tetrakis-1,3-benzenedisulfonic acid (100 nM), and 8',8',8',8'-[carbonylbis(mimo-3,1-phenylene-carbonylimino)]bis[1,3,5-naphthalenetrisulfonic acid (10 μM) significantly inhibited the outflow of [3H]noradrenaline, evoked by ATP, whereas Brilliant Blue G (100 nM), 2'-deoxy-Nβ-methyladenosine 3',5'-bisphosphate tetrammonium (10 μM), the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (250 nM), and the A2A receptor antagonist 3,7-dimethyl-1-propargylxanthine (250 nM) were ineffective. Pretreatment with the Gi protein inhibitor pertussis toxin (2.5 μg/ml) did not change the effect of ATP on [3H]noradrenaline outflow. In contrast, a decrease in extracellular pH from 7.4 to 6.6 significantly attenuated the response by ATP. When extracellular Na+ was replaced by choline chloride and in the presence of the noradrenaline uptake inhibitor desipramine (10 μM), the ATP-evoked [3H]noradrenaline outflow was almost completely abolished, indicating that its underlying mechanism is the sodium-dependent reversal of the noradrenaline transporter. Reverse transcription-polymerase chain reaction analysis revealed that mRNA encoding P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, and P2Y1, receptor subunits were expressed in the brainstem containing catecholaminergic nuclei projecting to the hippocampus, whereas mRNA encoding P2X3, P2X7, P2Y4, and P2Y6 receptors were absent. Taken together, these results indicate that noradrenergic terminals of the rat hippocampus are equipped with presynaptic facilitatory P2X receptors, displaying a pharmacological profile similar to homomeric P2X1 and P2X3 receptors.

ATP is released together with noradrenaline (Sperlágh et al., 1998; Poelchen et al., 2001) and acts as a cotransmitter in the sympathetic nervous system and at certain transmission sites of central catecholaminergic pathways (Poelchen et al., 2001). The action of ATP—either postsynaptic or presynaptic—is conveyed by P2 receptors, which are subdivided into ionotropic P2X and metabotropic P2Y families, consisting of seven (P2X1−7) and eight (P2Y1,2,4,6,11–14) individual receptor subunits (Chambers et al., 2000; von Kugelgen and Wetter, 2000; North, 2002). P2X receptors function as homo- or heterooligomeric coassemblies of various subunits forming non-selective cation channels, and their different combinations result in at least 18 different functional phenotypes (Torres et al., 1999). Among them, 12 individual coassemblies (homomeric P2X1−7 and heterooligomeric P2X1/2, P2X1/5, P2X2/3, P2X2/4, P2X3, P2X4, and P2X5) have been characterized pharmacologically in recombinant systems (Brown et al., 2002; North, 2002). P2Y receptors possess the traditional structure of G-protein-coupled receptors with seven transmembrane domains.
mains, intracellular N and extracellular C terminus, and they might also heteromerize with adenosine receptors but not with each other (Yoshioka et al., 2001). Adenosine receptors are another subfamily of G-protein-coupled receptors, sensitive to adenosine but not to ATP, having four molecularly identified members (A1, A2A, A2B, and A3; Fredholm et al., 2001).

Catecholaminergic nerve terminals are equipped with a wide variety of presynaptic auto- and heteroreceptors, including metabotropic α2-adrenergic, A1-adenosine, and P2Y nucleotide receptors that serve as sites of negative feedback modulation of noradrenaline release by the cotransmitters themselves (Boehm and Kubista, 2002). In our previous studies (Sperlah et al., 2000) and those of others (Boehm, 1999; Queiroz et al., 2003), it has been shown that the outflow of noradrenaline from sympathetic nerve terminals is also subject to modulation by facilitatory ionotropic P2X receptors. However, the pharmacological phenotype of these receptors varies between species, between transmission sites of the sympathetic nervous system, and even between the soma and nerve terminals of an individual neuron: e.g., in cultured sympathetic neurons of the rat, an α,β-methylene-adenosine 5′-triphosphate (α,β-methyleneATP)-insensitive P2X2-like receptor was identified (Boehm, 1999), whereas in our previous study, we found that α,β-methyleneATP powerfully stimulates noradrenaline outflow from the guinea pig right atrium, and the pharmacological profile of the underlying receptor was similar to that of P2X3 or P2X2/P2X3 receptors, consistent with the expression of their mRNA in the sympathetic ganglia (Sperlah et al., 2000). In a more recent study (Queiroz et al., 2003), the facilitatory P2X2 receptors involved in the modulation of noradrenaline outflow from the rat vas deferens were characterized, and the underlying receptors were identified as P2X2,-P2X2,- or P2X2/P2X3 receptors. In contrast, cultured mouse sympathetic nerve terminals do not seem to express a facilitatory nucleotide-sensitive receptor (Norenberg et al., 2001). Likewise, mRNA expression of different P2X receptor subunits also varies between species and different sympathetic ganglia within the same species (Dunn et al., 2001).

Less is known about the regulation of noradrenaline release by presynaptic P2 receptors in the central nervous system. Locus coeruleus neurons of the rat are equipped with ATP-sensitive P2X2 and P2X-like receptors, the activation of which facilitates the discharge of spontaneous action potentials (Frohlich et al., 1996). The outflow of noradrenaline from brain slices, including the hippocampus, is modulated by inhibitory A1 adenosine receptors (Jonzon and Fredholm, 1984) and P2Y-like receptors (Koch et al., 1997). On the other hand, it is not yet known whether activation of ionotropic P2 receptors also elicits noradrenaline release from central catecholaminergic pathways and if so, which purinoceptor subtype mediates this effect.

Therefore, to answer these questions, we examined the effect of P2 receptor activation on the outflow of [3H]noradrenaline from rat hippocampal slices, explored the mRNA expression of different P2 receptor subtypes in the brainstem containing catecholaminergic nuclei projecting to the hippocampus, and based on these data, an attempt was made to identify the underlying receptor subunits.

Materials and Methods

All studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and use of Laboratory animals and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary).

Experiments on [3H]Noradrenaline Outflow from Rat Hippocampal Slices. Tritium outflow experiments were carried out by the application of the method described in our earlier papers (e.g., Sperlah et al., 2000) to rat hippocampal slices. Briefly, male Wistar rats (140–160 g, Charles River Laboratories, Inc., Wilmington, MA) were anesthetized under light CO2 inhalation and then decapitated. The hippocampi were dissected in ice-cold Krebs’ solution saturated with 95% O2 and 5% CO2, and 400-μm-thick slices were prepared by a McIlwain tissue chopper and incubated in 1 ml of modified Krebs’ solution (113 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25.0 mM NaHCO3, and 11.5 mM glucose, pH 7.4) containing 370 kBq/ml [3H]noradrenaline ([3H]NA; 0.27 μM, specific activity 1.36 TBq/nmol; Amersham Biosciences Inc., Piscataway, NJ), ascorbic acid (30 μM), and Na2EDTA (100 μM) for 60 min. The medium was bubbled continuously with 95% O2 and 5% CO2 and maintained at 37°C. After loading, the slices were rinsed three times with 6 ml of Krebs’ solution, transferred to polypropylene tissue chambers, and superfused continuously with 95% O2 and 5% CO2-saturated modified Krebs’ solution (37°C, flow rate: 0.7 ml/min) containing ascorbic acid (30 μM) and Na2EDTA (100 μM) for 60 min. Subsequently, perfusate samples were collected over a 3-min period and assayed for tritium. Preparations were subjected to two identical periods of electrical field stimulation (EFS1 and EFS2; 25 V, 1 ms, 2 Hz, 240 shocks) delivered by a Grass S88 stimulator (Grass Instruments, Quincy, MA) or to two 6-min perfusion of agonists (ATP, ADP, and α,β-methyleneATP) with a 30-min interval in the absence or presence of drugs [pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), Brilliant Blue G, 8,8′-dimethyl-1,3-dipropylxanthine (DPCPX), 3,7-dimethyl-1-propargylxanthine (DMXPA), tetrodotoxin (TTX), D,L-2-amino-5-phosphono pentanoic acid (AP-5), 6-cyano-7-nitroquinolinoxaline-2,3-dione-disodium (CNQX), and desipramine], which were preperfused 18 min before the second agonist application. Concentration-response curves to P2 agonists were obtained upon tritium release elicited by S1. Since it is known that the high concentrations of ATP used in this study significantly acidify the dissolving medium (Sperlah et al., 2002), the pH of the ATP-containing perfusing solution was routinely adjusted to pH 7.4, except when the effect of the low pH was tested: in these experiments, the pH of the ATP-containing buffer was adjusted to pH 6.5. Likewise, a millimolar concentration of ATP alters the Ca2+ concentration of the medium, which—by changing the excitability of the membranes—might elicit transmitter release by itself. Nevertheless, this was not the case in our experiments, because restoring the Ca2+ concentration to 2.5 mM did not significantly alter ATP-evoked tritium outflow (data not shown). In some experiments, the slices were preincubated with the G protein inhibitor pertussis toxin (2.5 μg/ml) for 12 h before and during the loading period, and for respective controls, an identical period of preincubation was performed with drug-free Krebs’ solution. In other experiments, Ca2+-free Krebs’ solution or Na+-free Krebs’ solution supplemented with equimolar choline chloride was perfused for 60 min during the whole preperfusion period and thereafter to obtain complete replacement of unbound Ca2+ or sodium.

The radioactivity released from the preparations was measured with a Packard 1900 Tricarb liquid scintillation spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA), which is equipped with the Dynamic Color Corrected DPM Option providing
absolute activity calculation and correction for all kinds of color quenching. A 0.5-ml aliquot of the perfusate sample was added to 4 ml of liquid scintillation fluid (Packard ULTIMA GOLD), and counts were determined. For determining the residual radioactivity, the tissues were weighed and homogenized, and the radioactivity was extracted with 10% trichloroacetic acid. The counts were converted to absolute activity by the external standard method. Outflow of tritium was expressed in becquerels per gram and as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release). When the effects of different treatments and drugs on basal effluent were evaluated, the fractional release measured in a 3-min sample collected immediately after the second ATP perfusion (the 12th sample) were taken into account in the absence and presence of drugs, respectively. Agonist-induced tritium outflow (S1 and S2) was expressed by calculating the net release in response to agonist application by the area under the curve method, i.e., by subtracting the release before the agonist application from the values measured after agonist application, and a similar method was applied to calculate electrical field stimulation-evoked tritium outflow (EFS1 and EFS2). The effect of drugs on the agonist-evoked outflow of tritium was referred to S2/S1 ratios measured in the absence and presence of the drug, respectively.

HPLC Analysis. The composition of the tritium label in the hippocampal superfusate fluid was determined by a Gilson HPLC system using online enrichment with electrochemical and liquid scintillation detection, as described earlier (Sáhneta et al., 2000). The effluent contained [3H]NA and its [3H]-labeled metabolites, i.e., [3H]adrenaline, [3H]dihydroxyphenylethylene glycol, and [3H]normetanephrine. Therefore, the overall outflow of [3H]NA under various conditions can be regarded as an indicator of the outflow of both [3H]NA and its radioactive metabolites; however, for the sake of simplicity, we will refer to tritium outflow as [3H]NA outflow.

RT-PCR Study. Male Wistar rats (140-160 g) were decapitated under light CO2 anesthesia, and the brain was quickly put into ice-cold Krebs’ solution oxygenated with 95% O2 and 5% CO2. The brainstem was rapidly dissected and collected in liquid nitrogen.

Total RNA from brainstem samples was isolated with TriPure Isolation Reagent according to the protocol provided by the supplier (Roche Diagnostics, Indianapolis, IN). RNA samples dissolved in diethyl pyrocarbonate-treated water were checked on formaldehyde–agarose gels, and the identity of the amplified cDNAs and PCR products with the expected size has previously been verified by agarose gel electrophoresis. The identity of the amplified cDNAs and PCR products was then confirmed by sequencing (Sperlah et al., 2003). Genomic DNA contamination in the RNA samples was ruled out by direct PCR amplification of RNA samples.

Drugs. [3H]NA was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). The other drugs used were the following: ADP, ATP, a,b-methyleneATP, CNQX, desipramine, pertussis toxin, PPADS, TTX (all from Sigma-Aldrich, St. Louis, MO), AP-5, DMNX, DPCPX (Sigma/RBI, Natick MA), MRS2179, NPF23, and NPF449 (Tocris Cookson Inc., Bristol, UK). All solutions were freshly prepared on the day of use.

Statistics. All data were expressed as means ± S.E.M. of n observations. The statistical analyses were made by one-way analysis of variance followed by Dunnett’s or Tukey post hoc test (multiple comparisons) or Student’s t test (pair-wise comparisons). P values of less than 0.05 were considered statistically significant.

Results

The overall radioactivity uptake of the slices was 7.32 ± 0.35 × 105 Bq/g (n = 12). After 60-min preperfusion, the basal [3H]NA outflow measured in a 3-min sample was 0.57 ± 0.04% (n = 12) of tissue tritium content, which remained relatively constant during the subsequent sample collections. Electrical field stimulation (2 Hz, 1 ms, 240 shocks) elicited a rapid but transient elevation of [3H]NA outflow (EFS1: 2.20 ± 0.19%, n = 4). Six-minute perfusion of the preparations with ATP (10 mM) caused a similar increase in the outflow of [3H]NA which peaked 6 min after ATP administration and then gradually declined and returned to the baseline level in the following 12 min (Fig. 1). The net [3H]NA outflow evoked by ATP (10 mM) was 0.93 ± 0.20% (n = 12). A subsequent, identical challenge of ATP released a slightly lower amount of [3H]NA, resulting in a S2/S1 ratio of 0.80 ± 0.04 (n = 12) under control conditions.

The [3H]NA outflow evoked by ATP was concentration dependent between 1 and 30 mM (Fig. 2). Within this range, a clear maximal response was not obtained; therefore, EC50 values could not be calculated. HPLC analysis of the tritium label showed that the amount of [3H]NA significantly increased in response to ATP (from 0.25 ± 0.03% to 0.60 ± 0.023% of radioactivity remained in the tissue, n = 8, P < 0.001); on the other hand, the composition of the tritium label in the effluent did not significantly change, indicating that ATP released [3H]NA from the same pool that is mobilized under basal conditions (Table 1). Besides ATP, other ATP analogs, known to act as agonists on P2 receptors, were also effective in eliciting concentration-dependent [3H]NA outflow (Fig. 2); among them, a,b-methyleneATP was substantially more potent than ATP itself. ADP exerted similar response to

![Fig. 1. ATP-induced outflow of [3H]NA from rat hippocampal slices.](Image)

Hippocampal slices were superfused with Krebs’ solution for 60 min and then subjected to two 6-min (10 mM) ATP perfusions with 30-min intervals, as indicated by the horizontal bars. The tritium content in the perfusates was measured by liquid scintillation spectrometry and was expressed in fractional release (percentage; for calculation, see Materials and Methods), as a function of time. Data represent the means ± S.E.M. of 12 identical experiments.
The lengths of P2X-1-, P2X-2-, P2X-3-, P2X-4-, P2X-6-, P2X-7-, and P2Y-1-specific PCR products were as expected, indicating the expression of these P2 receptor subtypes. RT-PCR analysis showed that the sizes of the P2X-1, P2X-2, P2X-3, P2X-6, P2X-7, and P2Y-1-specific amplification products were consistent with the expected sequence-based product sizes, indicating the expression of these P2 receptor subtypes in the rat brainstem (Fig. 3). Their veracity was confirmed in previous studies by sequencing of the amplified PCR products (Sperla et al., 2003).

When PCR was performed on RNA samples without reverse transcription, there were no products, indicating that the template for amplification was the mRNA rather than the contaminating genomic DNA (data not shown). Amplification of β-actin was used as an internal control. Among P2X receptor subunits, mRNA encoding the P2X-3 subunit was below the detection limit, whereas among P2Y receptors, we could not detect the expression of P2Y-2, P2Y-4, and P2Y-11 receptors (Fig. 3). In our previous study (Sperla et al., 2003), using the same primer sets, mRNAs encoding all these subtypes were expressed in the rat hippocampus, which can be regarded as a positive control for the present experiments. mRNA expression of P2Y-11, P2Y-12, P2Y-13, and P2Y-14 receptors was not examined in the present study.

Next, the effect of ATP on [3H]NA outflow was tested in the presence of a variety of P2 receptor antagonists (Table 2). None of the antagonists significantly changed the basal [3H]NA outflow from the hippocampus (data not shown). PPADS (30 μM), the P2 receptor antagonist with wide subtype selectivity, significantly decreased the response evoked by ATP (10 mM). NF023, an antagonist of the P2X-1 receptor (Soto et al., 1999), also reduced [3H]NA outflow evoked by 10 mM ATP at 10 μM concentration but not at the P2X-1-selective 1 μM concentration (Table 2). However, NF449, another, more potent antagonist of P2X-1 receptors (Braun et al., 2001), profoundly reduced ATP-evoked [3H]NA outflow at 100 nM concentration (Table 2). On the other hand, the P2X-7 receptor antagonist with high selectivity, prevented the ATP-induced [3H]NA outflow (Table 2).

Table 1

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<tr>
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<th>Resting</th>
<th>ATP Induced</th>
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<tbody>
<tr>
<td>Noradrenaline</td>
<td>33.98 ± 2.65</td>
<td>34.85 ± 2.53</td>
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<tr>
<td>Adrenaline</td>
<td>15.43 ± 1.35</td>
<td>18.69 ± 1.95</td>
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<tr>
<td>Normetanephrine</td>
<td>32.45 ± 3.68</td>
<td>25.08 ± 2.17</td>
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<tr>
<td>[3H]Dihydroxyphenylethylene glycol</td>
<td>18.13 ± 2.27</td>
<td>21.36 ± 2.74</td>
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ATP at the lower concentration range, whereas at higher concentration, it elicited a clearly smaller amount of [3H]NA outflow, indicating that it has a lower potency than ATP (Fig. 2).

ATP and its analogs might act on a variety of P2 receptors belonging to the ionotropic P2X and G protein-coupled metabotropic P2Y receptor families. To examine the involvement of different subtypes of P2 receptors in the effect of ATP, at first mRNA expression of P2X and P2Y receptor subunits was examined in the brainstem, the area containing the cell bodies of central catecholaminergic neurons innervating the hippocampus. Total RNA samples from these brain regions were reverse transcribed and amplified by PCR using primers specific to different P2X and P2Y receptor subtypes. RT-PCR analysis showed that the sizes of the P2X-1, P2X-2, P2X-3, P2X-6, P2X-7, and P2Y-1-specific amplification products were consistent with the expected sequence-based product sizes, indicating the expression of these P2 receptor subtypes in the rat brainstem (Fig. 3). Their veracity was confirmed in previous studies by sequencing of the amplified PCR products (Sperla et al., 2003).
receptor selective antagonist Brilliant Blue G (100 nM; Jiang et al., 2000) was without effect (Table 2). To further delineate the P2X receptor subunits involved in the action of ATP, the effect of the change of extracellular pH was examined. It is known that ionotropic P2X receptors display differential sensitivity to the change of pH: the majority of the receptors, such as P2X$_4$ and P2X$_3$, decrease their activity at lower pH, whereas others, such as P2X$_2$ and P2X$_{3,6}$, increase their activity under these conditions (North, 2002). In our experiments, reduction of the pH of the Krebs’ solution from 7.4 to 6.5 decreased the response by ATP (10 mM) to less than one-half (1.29 ± 0.03% and 0.54 ± 0.11% at pH 7.4 and 6.5, respectively, n = 4, P < 0.01).

Since mRNA encoding P2Y$_2$ receptors was also expressed in the brainstem (see Fig. 3), one might assume that it could be also responsible for the effect of ATP analogs. Nevertheless, the P2Y$_2$, receptor-selective antagonist MRS2179 (10 μM) (Boyer et al., 1998), was ineffective to modify ATP (10 mM)-induced [H]$^3$NA outflow. To examine the involvement of A$_1$ and A$_2A$ adenosine receptors as well as the heteromeric P2Y$_2$/A$_1$ receptors in the ATP-induced [H]$^3$NA outflow, the effect of adenosine receptor antagonists were also tested. Nevertheless, DPCPX (250 nM), the A$_1$ receptor-selective antagonist, or DPX (250 nM), a nonselective adenosine receptor antagonist also acting at A$_2A$ receptors, did not affect [H]$^3$NA outflow induced by 1 mM ATP (Table 1). In accord with the previous observation, 12-h pretreatment of the slices with the G$_i$ protein inhibitor pertussis toxin (2.5 μg/ml) did not significantly modify ATP (10 mM)-evoked [H]$^3$NA outflow (1.13 ± 0.21% and 1.36 ± 0.33% after 12 h preincubation in the absence or presence of pertussis toxin, respectively, n = 4–8, P > 0.05). Therefore, metabotropic purine receptors, at least those which are coupled with G$_i$ protein, do not seem to participate in this effect.

ATP might elicit its effect on noradrenergic nerve terminals directly or indirectly by the activation of P2X receptors located on other neurons on non-neuronal cells in the hippocampus. To test the involvement of the main excitatory transmitter glutamate, the effect of glutamate receptor antagonists was examined. AP-5 (10 μM), a selective antagonist of NMDA-type glutamate receptors, did not significantly decrease ATP (10 mM)-evoked [H]$^3$NA outflow (Fig. 4A). When CNQX (30 μM), an antagonist of the non-NMDA receptors, was also included to the perfusion medium, a small, additional reduction of [H]$^3$NA outflow by ATP was observed, which reached the level of significance when compared with the control group (Fig. 4A), indicating that the effect of ATP is partly mediated by endogenous glutamate receptor activation. On the other hand, the voltage-dependent sodium channel blocker tetrodotoxin (1 μM) was without significant effect, indicating that axon potential propagation has only minor, if any, role in this effect (Fig. 4A). Omission of [Ca]$^{2+}$ from the Krebs’ solution significantly increased the basal outflow of [H]$^3$NA (0.67 ± 0.04% n = 8, P < 0.01 versus control: 0.44 ± 0.04%, n = 12) and almost entirely inhibited electrical field stimulation-evoked [H]$^3$NA outflow (EFS; 0.12 ± 0.04%, n = 4, P < 0.001 versus control). The [H]$^3$NA outflow elicited by ATP, however, appeared to be [Ca]$^{2+}$-independent because no change in its amount was observed.
after 60-min preperfusion with Ca$$^{2+}$$-free Krebs’ solution (Fig. 4B). On the other hand, replacement of the sodium in the perfusion buffer with equimolar choline chloride, although it increased basal [3H]NA outflow (1.07 ± 0.25%, n = 4, P < 0.01), it completely abolished the ATP (10 mM)-evoked [3H]NA outflow (Fig. 4B). The noradrenaline uptake inhibitor desipramine (10 µM) by itself did not change the basal [3H]NA outflow (0.40 ± 0.02%, n = 4, P > 0.05) but also inhibited ATP-evoked [3H]NA outflow (Fig. 4B).

Discussion

In this study, we explored the facilitatory effect of P2 receptor activation on the outflow of [3H]NA from the rat hippocampus, which receives a dense noradrenergic projection arising from the locus coeruleus (Oleskevich et al., 1989). ATP evoked a concentration-dependent increase in basal [3H]NA efflux, which was reversible and reproducible upon a second identical stimulus. To explore the expression of P2X and P2Y receptor subunits, potentially contributing to the effect of ATP to release [3H]NA from hippocampal slices, an RT-PCR analysis was performed in the brainstem, containing the cell bodies of locus coeruleus noradrenergic neurons. The analysis revealed a nonuniform distribution of the mRNA encoding various P2X and P2Y receptors, showing a positive signal in the case of P2X1, P2X3, P2X4, and P2Y1, but the signal was below the detection limit in the case of P2X5 and P2Y2,4,6 receptors. Our results are in good agreement with a previous study (Collo et al., 1996) reporting the expression of P2X2,4,6 but not P2X5 in locus coeruleus of the rat brain. On the contrary, Collo et al. (1996, 1997) and other earlier reports (e.g., Kidd et al., 1995) could not find expression of mRNA encoding P2X2, P2X3, and P2X7 receptors in the rat brain by in situ hybridization, but this divergence can be explained by the higher sensitivity of the RT-PCR technique. As for P2Y receptors, mRNA expression of P2Y1 receptors was evaluated in rat (Moran-Jimenez and Matute, 2000), chick (Webb et al., 1998), and human (Moore et al., 2001) brain, and all three studies found consistent expression in brainstem regions including the locus coeruleus, which is in line with our results. Fewer data are available on the mRNA expression of P2Y2, P2Y4, and P2Y6 receptors, but all studies found low or no expression in the chick (Webb et al., 1998) and human (Moore et al., 2001) brain. It is necessary to note, however, that the presence of mRNA encoding individual P2X and P2Y receptor subtypes in the whole brainstem does not prove their expression in the locus coeruleus noradrenergic neurons, and mRNA levels do not necessarily reflect the expected level of functional protein expression. Therefore, the purine receptor subtype responsible for the facilitation of [3H]NA outflow by ATP analogs was also dissected pharmacologically.

As for the agonist profile ATP and its metabolically stable analog α,β-methyleneATP, both were effective in releasing [3H]NA from hippocampal slices, the latter being the more potent, whereas ADP also acted as an agonist with lower potency than ATP. Among P2X receptors with known pharmacological profile, P2X1, P2X3, P2X4, P2X5, P2X7, and P2X10 receptors are activated by α,β-methylene ATP; the low level of mRNA encoding P2X5 receptor subunits in the brainstem, however, renders unlikely the involvement of P2X5 receptors. As for the P2X4 receptor, α,β-methyleneATP is only a partial agonist, and its maximal response is less than 12% of that caused by ATP (Le et al., 1998); therefore, it might not be involved in this effect. Although in general ATP is more potent than α,β-methyleneATP at recombinant P2X receptors, one might assume that the very low potency of ATP in our experiments is due to the rapid and highly effective breakdown of extracellular ATP in the hippocampus (Cunha et al., 1998): to the feed forward inhibition of ectonucleotidases by nucleotides, which is not apparent at lower doses (Cunha, 2001); and to the simultaneous activation of inhibitory P2Y and A1 adenosine receptors. Although α,β-methyleneATP, which also acted in higher concentrations than in recombinant systems, is also subject to localized catalysis in this brain area, its hydrolysis rate is slower. On the other hand, antagonists of P2X receptors inhibited the effect of ATP in lower concentration range, even in nanomolar concentration (NF449), which implies that the low potency of ATP and its analogs cannot be explained by drug penetration problems. In our experiments, ADP appeared to be less potent than ATP because its effect at the highest concentration tested (30 mM) was less than that of elicited by ATP.

The ATP-evoked [3H]NA outflow was significantly decreased by the P2 receptor antagonists PPADS, NF023, and NF449, but not by Brilliant Blue G and MRS2179. This profile is consistent with the involvement of P2X1, P2X4, and P2X5 receptors. However, the lack of significant effect by NF023 at the lower P2X1-selective concentration indicates that the P2X5 or P2X2/3 rather than the P2X1 receptor is responsible for this effect. On the other hand, NF449, which is another potent antagonist of P2X1 receptors (Braun et al., 2001), powerfully inhibited the effect of ATP at 100 nM concentration, although this concentration is higher than its affinity to the P2X1 receptor but is still below the concentration necessary to inhibit the P2X2/3 receptor. Therefore, the involvement of P2X2/3 receptors or other unidentified heteromeric coassembly P2X receptor subunits in this effect cannot be entirely excluded.

Since it is known that lowering the pH differentially affects the response mediated by P2X receptor subunits, the effect of a change in the extracellular pH was tested. Lowering the extracellular pH from 7.4 to 6.5 significantly inhibited ATP-evoked [3H]NA outflow, which is in agreement with the involvement of P2X1 and P2X5 receptors but argues against the involvement of the P2X2/3 receptor, which responds to the acidic environment with potentiation. For the same reason, a role for the newly identified P2X17/18 combinations also seems unlikely (Brown et al., 2002).

Besides P2X receptors, ATP-evoked [3H]NA outflow could also be mediated by other nucleotide-sensitive receptors, i.e., the P2Y subfamily, and among them mRNA encoding the P2Y1 receptor, but not P2Y2, P2Y4, and P2Y6, was present in the brainstem. However, MRS2179, a selective antagonist of P2Y1 receptors (Boyer et al., 1998), did not significantly affect the action of ATP. Although we have not explored the mRNA expression of the four newly identified P2Y receptor subtypes, i.e., P2Y11, P2Y12, P2Y13, and P2Y14, our pharmacological findings do not support their involvement in the action of ATP for the following reasons: 1) although α,β-methyleneATP is also an agonist of the P2Y11 receptor, this receptor is insensitive to PPADS, which acts as an antagonist in our experiments; 2) according to the present knowl-
edge, P2Y12 and P2Y13 are coupled to G proteins, but inhibition of G proteins with pertussis toxin did not affect ATP-evoked [3H]NA outflow (Hollopeter et al., 2001; Zhang et al., 2002), and furthermore, ATP acts as an antagonist at P2Y12 receptor; and 3) the P2Y14 receptor (Chambers et al., 2000) is not activated by either ATP or ADP.

Finally, A1 and A2A adenosine receptors as well as the heteromeric P2Y1/A1 receptors were taken into account. Nevertheless, DPCPX and DMPX, antagonists of these receptors, were inactive in inhibiting the response elicited by ATP.

We also identified the underlying mechanism responsible for the effect of ATP in our experiments. Being unaffected by TTX, action potential propagation seems to play a minor role in this effect. On the other hand, the activation of glutamate receptors elicits noradrenaline release from the hippocampus (Pittaluga and Raiteri, 1992); therefore, we explored the role of endogenous glutamate release and subsequent glutamate receptor activation in this effect. When NDMA and non-NDMA receptors were completely inhibited by a cocktail of AP-5 and CNQX, a small but significant attenuation of the effect of ATP was observed, showing that local or nonsynaptic glutamate receptor activation might be partly involved in this response. Omission of Na+, but not Ca2+, from the perfusion medium abolished ATP-evoked [3H]NA outflow, which implies that the mechanism responsible for the action of ATP is a direct influx of sodium through the receptor ion channel complex. Although P2X receptor-gated ion channels are also permeable to Ca2+, in the absence of Ca2+, Na+ might substitute Ca2+ to promote transmitter efflux. In the reverse situation, i.e., in the absence of Na+, this might not be true, because [Na+]o is much higher than [Ca2+]. Moreover, the [3H]NA outflow evoked by ATP was completely abolished by the noradrenaline transporter inhibitor desipramine, which indicates that [3H]NA released from the cytoplasm by the Na+-dependent reversal of the noradrenaline transporter. This is also consistent with the Ca2+-independent nature of [3H]NA outflow evoked by ATP and with the findings that noradrenaline and its metabolites were also present in the effluent, suggesting the cytoplasmic origin of the released tritiated substrates. Since desipramine does not block noradrenaline noradrenergic transporters, its powerful effect on ATP-evoked [3H]NA outflow is indicative of a neuronal release.

In conclusion, the activation of P2X receptors elicits [3H]NA outflow from the rat hippocampus, and the most likely candidates responsible for this action are the homomeric P2X1 or P2X2 receptors. This arrangement is similar to that found recently in rat sympathetic nerve terminals, where P2X1, P2X3, and P2X2/3 receptors were identified to facilitate noradrenaline release from slices of the rat hippocampus. Life Sci 55:1971–1977.


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