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Ligand Recognition Sites on P_{2X} Receptors Studied by Quantitative Autoradiography of [^{3}H]α,β-Methylene-ATP Binding in Rat Brain

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The specificity of α,β-methylene-ATP for P_{2X} receptor binding sites in the CNS has been examined by testing the effects of several ATP analogues and other ATP-related substances on the binding of 10 nM [^{3}H]α,β-methylene-ATP to 20 μm thick sections of fresh-frozen rat brain. The labelling of the putative P_{2X} receptor binding sites by [^{3}H]α,β-methylene-ATP was evaluated by quantitative densitometry. [^{3}H]α,β-Methylene-ATP binding was strongly inhibited by two close ATP analogues, 3′-O-(trinitrophenyl)-adenosine-5′-triphosphate and β,γ-imido-ATP (IC_{50} 2-5 μM). β,γ-Methylene-ATP was, however, less potent (<50% inhibition at 25 μM). Inosine-5′-triphosphate, guanosine-5′-triphosphate, uridine-5′-triphosphate, and cytidine-5′-triphosphate were practically inactive up to concentrations of 100 μM. Periodate oxidised ATP and 1,N^6-etheno-ATP produced <50% inhibition at 100 and 500 μM concentrations, respectively. Cations (K^+, Rb^+, Cs^+, and Mg^{2+} at 5 mM and Na^+ at 150 mM) reduced [^{3}H]α,β-methylene-ATP binding by no more than 50%. Several agents known to interact with Ca^{2+}- and/or ATP-related cationic channels (Cd^{2+}, glibenclamide, dantrolene, nifedipine, and thapsigargin) had no effect. We conclude that [^{3}H]α,β-methylene-ATP at low nanomolar concentrations binds to a site that has very strict structural requirements and is pharmacologically similar to ATP P_{2X} receptors.

Rapid excitatory actions of extracellular ATP in the central and peripheral nervous systems and in smooth muscle are mediated by receptors of the P_{2X} class [1,2,3, for a review see 4]. P_{2X} receptors act as fast ligand-gated cationic channels located in the cytoplasmic membranes of neurons, myocytes and possibly fibrocytes and glial cells [5]. Recent advances in the molecular biology and protein chemistry of P_{2X} receptors [for reviews see 6,7] have greatly facilitated the development of antibodies directed against individual subunits of P_{2X} receptors [7,8,9]. However, while immunocytochemistry can visualize the location of these P_{2X} receptor subunits it cannot provide information about the biochemical and pharmacological characteristics. Such data can be readily obtained by radioligand binding techniques using frozen sections of brain and spinal cord [10]. If quantitative densitometry is used to evaluate the autoradiographical data [11,12], the properties of P_{2X} receptor-associated binding sites can be established *in situ* and their variations can be studied across the major regions of the central nervous system (CNS). The only radioligand so far used in the characterization of P_{2X} binding sites is the ^{3}H-labelled form of the stable ATP analogue [^{3}H]α,β-methylene-ATP [13,14]. This compound differentiates P_{2X} from P_{2Y} receptors [15,16] and has been successfully employed as a radioligand in autoradiographic studies [10,11,12,17]. Expression-cloning of P_{2X} receptors has, however, indicated that some of the subunits are relatively insensitive to α,β-methylene-ATP [18] and, for this and related reasons, specificity of [^{3}H]α,β-methylene-ATP as a P_{2X} radioligand has been questioned [19]. In the present autoradiographic experiments we have examined, as potential inhibitors of [^{3}H]α,β-methylene-ATP binding, ATP analogues, cations (Na^+, K^+, Rb^+, Cs^+ and Mg^{2+}) and several compounds known to interact with sites, other than P_{2X} receptors, which could bind ATP. We discuss the characteristics of the α,β-methylene-ATP binding site in frozen brain sections and evaluate advantages and limitations of using [^{3}H]α,β-methylene-ATP as a P_{2X}-specific radioligand in quantitative autoradiographic studies.

MATERIALS AND METHODS

Methods have been described in detail previously [11,12,20,21]. Brains from Sprague-Dawley rats, 3-6 months of age, either sex,

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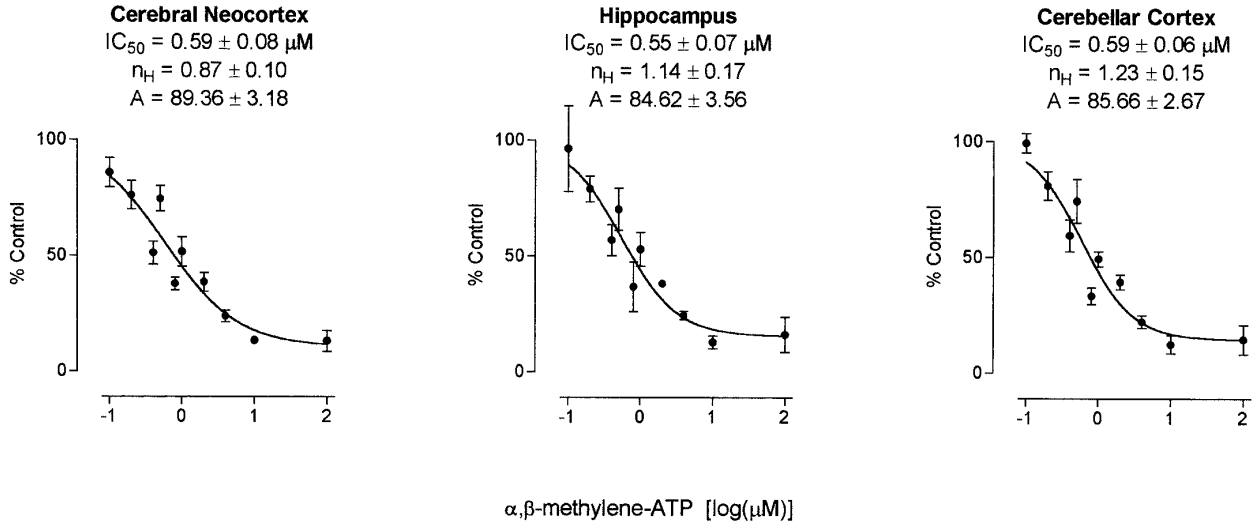

FIG. 1. The data were fitted to the equation I = 100 − A/(1 + 10∧(n_H*log(IC_{50}) − X) where A is a portion of binding of 10 nM [^{3}H]α,β-methylene-ATP resistant to the inhibition by non-labelled α,β-methylene-ATP and X is $\log_{10}$[inhibitor concentration] (modified from [34]). The F-test [34] indicated that the above equation provided a better fit than a similar equation in which A = 100 ($P < 0.001$). The value of A actually corresponds to "specific" binding, which was determined in all subsequent experiments directly by subtracting [^{3}H]α,β-methylene-ATP binding in the presence of 10 μM non-labelled α,β-methylene-ATP from the value of total binding.

decapitated under 5% halothane/N_2O anaesthesia, were submerged in Tissue Tek (Miles, USA), rapidly frozen and stored at −20 °C. Within 2 weeks 20 μm thick sections were prepared and thaw-mounted on gelatine-coated slides. The sections were stored, usually 2 to 3 days, at −20 °C before being used in experiments. On the day of the experiment, the sections were first hydrated using 50 mM Tris-HCl medium (containing 2.5 mM Ca^{2+}, pH 7.4) at room temperature and then incubated at 2-4 °C, using medium of the same composition, in the presence of 10 nM [^{3}H]α,β-methylene-ATP. Binding in the presence of 10 μM nonradioactive α,β-methylene-ATP was considered nonspecific. At the end of the experiments, the sections were rapidly washed, dessicated and subsequently exposed for 10 days against tritium-sensitive film (Hyperfilm-^{3}H, Amersham International plc., UK).

Autoradiograms were scanned by a laser beam (1 mV, HeNe, wavelength 632.8 nm; Molecular Dynamics Personal Densitometer), analysed by ImageQuant software (Molecular Dynamics) and the results were converted, using tritium standards (^{3}H-Microscales, Amersham International plc., UK) into fmol/mg wet weight [12]. Statistical analyses, including non-linear regression fits and ANOVA (Tukey-Kramer comparisons) were carried out as described previously [11,21] using Prism Software (GraphPad Inc., San Diego, CA, USA).

[^{3}H]α,β-Methylene-ATP was obtained from New England Nuclear, Boston, MA, USA, (specific activity 250 mCi, Lot No. 3248664), α,β-methylene-ATP, β,γ-methylene-ATP, β,γ-imido-ATP, α,β-methylene-ADP and guanosine triphosphate-γ-sulphate (GTPγS) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals originated from commercial suppliers and were of at least analytical grade.

RESULTS AND DISCUSSION

[^{3}H]α,β-Methylene-ATP produced an uneven labelling distributed over the brain regions as reported previously [11,12]. A typical dose-response relationship for an inhibition of [^{3}H]α,β-methylene-ATP binding by the nonradioactive α,β-methylene-ATP is shown in Fig. 1 and the summary of the effects of ATP analogues in selected brain regions is presented in Table 1. α,β-Methylene-ATP interacts with the [^{3}H]α,β-methylene-ATP-binding site significantly more strongly than its close analogue β,γ-methylene-ATP. This is in accordance with the proposal that α,β-methylene-ATP interacts preferentially with central neuronal P_{2X} receptors, whereas β,γ-methylene-ATP may be the preferred ligand for P_{2X} receptors on smooth muscle [22]. It has been speculated that the position of the methylene group in the triphosphate chain (Fig. 2) is crucial for the binding of Ca^{2+} which may be crucial for maintaining the affinity of the site for the ligand [12]. It is therefore, interesting to note that the β,γ-imido-ATP is apparently closer in its potency to α,β-methylene-ATP than to β,γ-methylene-ATP (Table 1). Evidently, substitution of the potentially ionizable imido group for the methylene carbon partly offsets the putative disadvantage imparted by the greater distance and altered angle between the β and γ as opposed to α and β phosphates. Both β,γ-methylene-ATP and 3′-O-(trinitrophenyl)-adenosine-5′-triphosphate (TNP-ATP) are weaker inhibitors of [^{3}H]α,β-methylene-ATP-binding in the cerebellar cortex than in forebrain structures (Table 1). The difference is not large, however, thus implying only subtle regional variations in the nature of [^{3}H]α,β-methylene-ATP-binding site in the CNS.

The present study confirms suggestions that minor modifications to the triphosphate chain of the ligand will have major effects on binding. The interaction of β,γ-imido-ATP with the binding site, which is stronger than that of β,γ-methylene-ATP, implies that the distances separating the phosphate groups may not be the only factor determining the affinity of a ligand for the

TABLE 1

Inhibition of [^{3}H]α,β-Methylene-ATP Binding by ATP Analogues

	Cerebral neocortex	Hippocampus	Cerebellar cortex
	IC_{50} ± SEM (μM) n_H ± SEM		
(a)			
α,β-methylene-ATP	0.59 ± 0.08 0.87 ± 0.10	0.55 ± 0.07 1.14 ± 0.17	0.59 ± 0.06 1.23 ± 0.15
(0.1–100 μM)	(n_c = 10, n = 52)	(n_c = 10, n = 47)	(n_c = 10, n = 53)
β,γ-imido-ATP	2.23 ± 0.13 1.36 ± 0.10***	2.73 ± 0.22 2.00 ± 0.29***	4.59 ± 0.37[a] 1.40 ± 0.16**
(0.5–100 μM)	(n_c = 10, n = 59)	(n_c = 6, n = 33)	(n_c = 9, n = 59)
FTP	23.5 ± 4.4 0.68 ± 0.11*	23.9 ± 0.2 0.69 ± 0.10*	20.2 ± 2.9 0.70 ± 0.11
(1.0–97 μM)	(n_c = 7, n = 45)	(n_c = 7, n = 45)	(n_c = 7, n = 45)
TNP-ATP	2.23 ± 0.39 0.65 ± 0.07***	3.74 ± 0.48 0.76 ± 0.07**	4.44 ± 0.55[b] 0.80 ± 0.16*
(1.0–100 μM)	(n_c = 7, n = 38)	(n_c = 7, n = 38)	(n_c = 7, n = 38)
	% Inhibition ± SD (concentration, n)		
(b)			
ATP-po	31 ± 6*** (100 μM, 6)	25 ± 10** (100 μM, 6)	34 ± 7** (100 μM, 6)
ITP	n.s.	32 ± 9* (100 μM, 5)	n.s.
GTPγS	35 ± 6*** (10 μM, 5)	35 ± 10* (10 μM, 3)	n.s.
ϵ-ATP	47 ± 3** (500 μM, 5)	44 ± 5* (500 μM, 4)	47 ± 8** (500 μM, 5)
	60 ± 3*** (1000 μM, 5)	60 ± 3** (1000 μM, 4)	63 ± 5*** (1000 μM, 5)
β,γ-Methylene-ATP	45 ± 5*** (25 μM, 6)	43 ± 3** (25 μM, 6)	39 ± 4** (25 μM, 6)
α,β-Methylene-ADP	40 ± 3*** (80 μM, 6)	39 ± 4** (80 μM, 6)	34 ± 1** (80 μM, 5)

Note. The concentration of [^{3}H]α,β-methylene-ATP was 10 nM in all experiments. In section a, compounds were tested over a concentration interval given in parentheses. The number of concentrations is given as n_c and n corresponds to the total number of experimental points. IC_{50} stands for the concentration of inhibitor causing 50% inhibition of specific binding (cf. Fig. 1) and n_H is the Hill slope coefficient (different from 1 at $P < 0.05$*, 0.01**, or 0.001*** by the F-test [35]). IC_{50} in the cerebellar cortex was different from that in the hippocampus and the cerebral neocortex at $P < 0.001^a$ and from that in the cerebral neocortex at $P < 0.01^b$, by ANOVA followed by the Tukey-Kramer test. In section b, inhibitions were statistically significant at $P < 0.05$*, 0.01**, or 0.001*** by ANOVA, followed by the Tukey-Kramer test (only the inhibitions measured at the highest concentration(s) of test compounds are presented). GTP, UTP, and CTP produced no statistically significant inhibitions up to 100 μM.

binding site. The ability of the phosphate chain to bind Ca^{2+} appears a more important factor for the strength of the bonding between the ligand and the binding site.

Amongst other analogues of ATP, neither cytidine-5′-triphosphate (CTP), uridine-5′-triphosphate (UTP), inosine-5′-triphosphate (ITP) nor guanosine-5′-triphosphate (GTP) produced strong inhibitions of [^{3}H]α,β-methylene-ATP binding (Table, see also legend). In fact, earlier studies using the same experimental conditions as used here have found that ATP analogues with substitutions at position 2 on the purine had no effect on [^{3}H]α,β-methylene-ATP binding [11]. Thus it seems that the intact structure of the adenine moiety is just as essential for the interaction with the [^{3}H]α,β-methylene-ATP binding site as is the conformation of the triphosphate chain.

In support of the conclusion that the adenine ring will tolerate virtually no structural alterations is the fact that the 1,N^6-etheno-ATP (ϵ-ATP) (Fig. 2) is largely inactive and even the formycin group of FTP (Fig. 2), with its slightly altered glycosylic bond angle, exhibits greatly reduced binding. The sugar moiety, however, is able to accommodate large substituents, particularly in the 2′ and 3′ positions such as the trinitrophenyl group (Fig. 2). The simplest interpretation of this finding is that the ribose moiety is oriented in such a way that the 2′ and 3′ positions face away from the binding site and the space in their vicinity is not occupied by any part of the binding protein or by a neighbouring structural component of the membrane. The periodate oxidized ATP clearly has a dramatically altered ribose ring structure which essentially precludes binding to the receptor.

It is interesting to note that the inhibition by β,γ-methylene-ATP displayed $n_H > 1$ (apparent positive cooperativity) while the inhibitions by compounds with altered ribose and purine rings (FTP, TNP-ATP) had $n_H < 1$ (apparent negative cooperativity). This implies that fundamental differences exist between the modes of binding of, respectively, analogues with altered phosphate chains and those with modifications in ribose or purine rings. Furthermore, $n_H \neq 1$ may indicate a multiplicity of [^{3}H]α,β-methylene-ATP binding sites. There are at least seven P_{2X} receptor subunits in mammalian tissue [6,7] and several of the subunits may contribute to the formation of homomeric/heteromeric P_{2X} channel complexes, thus producing a variety of [^{3}H]α,β-methylene-ATP sites, each with a characteristic pharmacological profile.

GTPγS binds strongly to G-proteins [23]. However, it

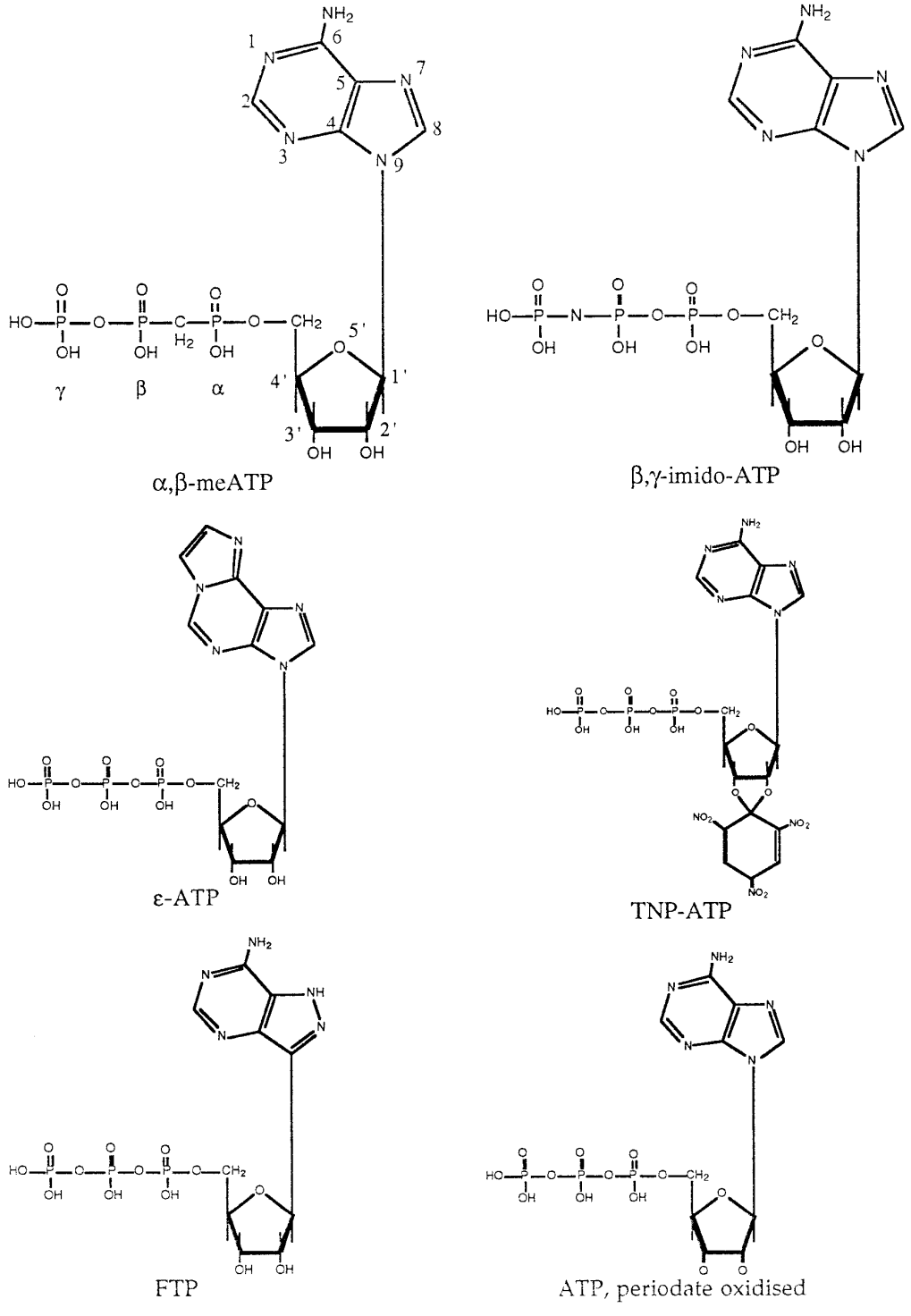

FIG. 2. Structural formulae of α,β-methylene-ATP, FTP, TNP-ATP, ϵ-ATP and periodate-oxidized ATP.

produced only a small inhibition of [^{3}H]α,β-methylene-ATP binding (about 35% inhibition at 10 μM in the cerebral neocortex and hippocampus, but no statistically significant inhibition in the cerebellar cortex, Table 1, see also legend). No enhanced effect of GTPγS was observed in the presence of 2.5 mM Mg^{2+}, which is normally essential for the interaction between GTP and G-proteins [23]. Neither the inhibitor of ATP-linked K^+-channels, glibenclamide [24,25], the L-calcium channel blocker, nifedipine [26,27], nor the

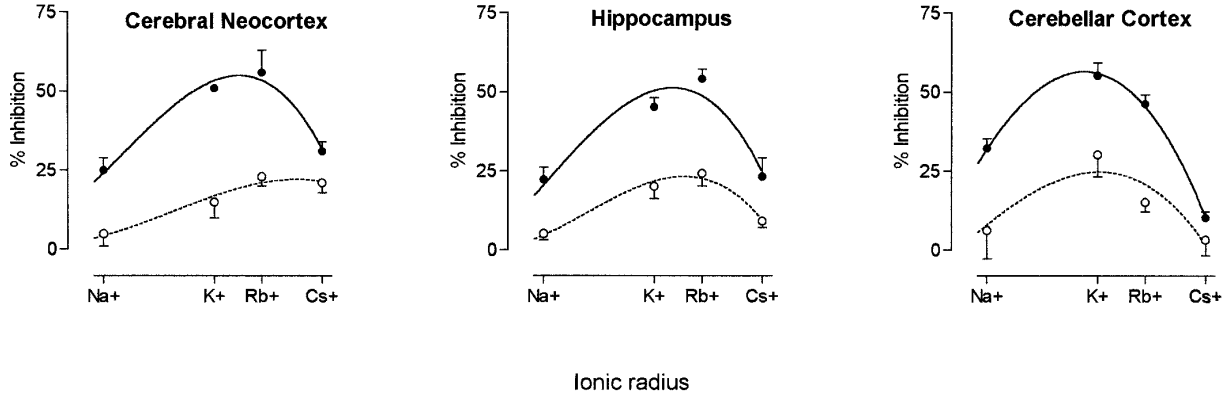

FIG. 3. Inhibitions (means ± SEM of 4-6 values) of the specific binding of 10 nM [^{3}H]α,β-methylene-ATP are plotted against the ionic radii of monovalent cations and fitted by a third-order polynomial. Full circles and solid lines correspond to 5 mM concentrations of ions, open circles and dotted lines correspond to 1 mM ion concentrations. Goldschmitt radii were used: Na^+, 98 pm; K^+, 133 pm; Rb^+, 148 pm; and Cs^+, 167. Li^+ (68 pm) produced no statistically significant inhibition at either 1 or 5 mM concentrations.

blocker of Ca^{2+} channels on the endoplasmic reticulum, dantrolene [28], had any effect at 10 μM concentrations. The potent irreversible inhibitor of Ca^{2+}-dependent ATPase in the endoplasmic reticulum, thapsigargin [29,30], produced <10% inhibition ($P < 0.05$) at a concentration of 40 μM. The cytoskeleton has been implicated in the regulation of voltage-dependent Ca^{2+} channels [31] but phalloidin, a stabilizer of the cytoskeletal microfilaments [32], had no effect (<10% inhibition at 35 μM, $P > 0.05$).

Present and previous data [11] indicate that the inhibitors of the most common ATPases and ligands for other cationic channels have little, if any, influence on the binding of [^{3}H]α,β-methylene-ATP. Furthermore, the greater affinity of α,β-methylene-ATP for the [^{3}H]α,β-methylene-ATP site, as compared with the affinity of β,γ-methylene-ATP, is consistent with the characteristics of the ligand binding site at P_{2X} receptors on neuronal membranes [22, *vide* 12 for the similar characteristics of [^{3}H]α,β-methylene-ATP binding in the sections of spinal cord].

Binding of [^{3}H]α,β-methylene-ATP was only marginally influenced by low mM concentrations of monovalent and divalent cations implying that either extracellular or intracellular cations have only a minor effect on the [^{3}H]α,β-methylene-ATP binding site (Fig. 3). The data indicate that there could be an "optimum" ionic radius, close to those of K^+ and Rb^+ at about 1.4 pm, that would best fit a putative binding site for monovalent cations, exhibiting a negative effect on the binding of [^{3}H]α,β-methylene-ATP. Alternatively, there could be two cation-binding sites: one preferring smaller monovalent cations and exerting a negative effect on the ligand binding; and a second site with a preference for larger ions, but promoting [^{3}H]α,β-methylene-ATP binding. Higher concentrations of Na^+ (150 mM, similar to the extracellular concentration) produced about the same inhibition of [^{3}H]α,β-methylene-ATP binding as 5 mM. The general blocker of voltage-dependent Ca^{2+} channels, Cd^{2+}, tested as 50 μM $CdCl_2$ had no effect. Binding of [^{3}H]α,β-methylene-ATP was, however, reduced in all three brain regions in the presence of Mg^{2+} (18-23% at 1 mM and 46-49% at 5 mM).

It has been suggested that [^{3}H]α,β-methylene-ATP may bind to a 5′-nucleotidase present in homogenized tissue [19]. However, 5′-nucleotidase prepared from mammalian brain tissue was shown to be strongly inhibited by α,β-methylene-ADP ($K_i < 1$ nM [33]). In our experiments α,β-methylene-ADP at 80 μM produced <50% inhibition. This suggests that α,β-methylene-ADP is a 3-4 orders of magnitude weaker inhibitor at [^{3}H]α,β-methylene-ATP-labelled sites than at the substrate binding sites of 5′-nucleotidase. Moreover, GTP, UTP and CTP, all known inhibitors of 5′-nucleotidase with K_i values <1 μM [33], had no effects on the [^{3}H]α,β-methylene-ATP-labelled sites. It is, therefore, very improbable that α,β-methylene-ATP binding sites on 5′-nucleotidase significantly contributed to the binding of [^{3}H]α,β-methylene-ATP observed in the present experiments.

In summary, the binding of [^{3}H]α,β-methylene-ATP to thaw-mounted sections of frozen rat brain has characteristics very different from those on ATPases and cationic channels. The interaction between [^{3}H]α,β-methylene-ATP and the binding site requires a specific configuration of the triphosphate side chain and an intact adenine moiety but substituents on certain parts of the ribose moiety do not significantly interfere with the binding.

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