0026-895X/97/030524-09$3.00/0

MOLECULAR PHARMACOLOGY, **51:**524–532 (1997).

Radiolabeling of the Rat $P2X_4$ Purinoceptor: Evidence for Allosteric Interactions of Purinoceptor Antagonists and Monovalent Cations with P2X Purinoceptors

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Received August 12, 1996; Accepted November 11, 1996

SUMMARY

The rat recombinant $P2X_4$ purinoceptor was expressed in CHO-K1 cells, and binding studies were performed using the radioligand [^{35}S]adenosine-5′-*O*-(3-thio)triphosphate ([^{35}S]ATPγS). In 50 mM Tris/1 mM EDTA assay buffer, pH 7.4 at 4°, [^{35}S]ATPγS bound with high affinity to the $P2X_4$ purinoceptor (K_D = 0.13 nM, B_{max} = 151 pmol/mg of protein). The purinoceptor agonists ATP and 2-methylthioadenosine triphosphate possessed nanomolar affinity for the $P2X_4$ purinoceptor, whereas the antagonist suramin possessed much lower affinity (IC_{50} = 0.5 mM). Cibacron blue was more potent than suramin but produced a biphasic competition curve, whereas *d*-tubocurarine potentiated binding at concentrations in excess of 10 μM. The complex effects of cibacron blue and *d*-tubocurarine seemed to be due to an allosteric interaction with the $P2X_4$ purinoceptor because these compounds affected radioligand dissociation, measured after isotopic dilution with unlabeled ATPγS. Cibacron blue (1–100 μM) and *d*-tubocurarine (0.1–1 mM) produced rapid (10 sec to 5 min) decreases or increases, respectively, in the level of [^{35}S]ATPγS binding measured immediately after initiation of the dissociation reaction. However, the subsequent rates of radioligand dissociation were not markedly different from those measured in their absence. Monovalent cations produced similar affects on the $P2X_4$ purinoceptor and, like *d*-tubocurarine, increased [^{35}S]ATPγS binding. The actions of *d*-tubocurarine and sodium were not additive. The findings from this study indicate that [^{35}S]ATPγS can be used to label the $P2X_4$ purinoceptor and suggest that this binding can be enhanced by monovalent cations and *d*-tubocurarine and may be subject to negative allosteric modulation to varying degrees by different purinoceptor antagonists.

The P2X purinoceptors represent a family of ligand-gated cation channel receptors for extracellular ATP of which at least seven different P2X purinoceptor genes have been identified so far (1–8). Each of the P2X purinoceptor genes encodes for subunits that when heterologously expressed, form fully functional homomeric P2X purinoceptor subtypes. In addition, there is evidence that the $P2X_2$ and $P2X_3$ purinoceptor subunits may heteropolymerize to form a novel P2X purinoceptor subtype (3). Although the recombinant receptors exhibit quite marked differences in functional studies, such differences are not readily discernible at a molecular level in receptor binding studies. Thus, although it is possible to label directly the recombinant $P2X_1$ and $P2X_2$ purinoceptors using the radioligand [^{35}S]ATPγS, the binding characteristics of these two receptors are very similar despite quite marked differences in their functional pharmacology (9, 10).

To understand better the interaction of ligands with P2X purinoceptors, the aim of this study was to determine whether [^{35}S]ATPγS could be used to label an additional member of the P2X purinoceptor family (ie, the $P2X_4$ purinoceptor). This receptor is of particular interest because in functional studies, it can be readily distinguished from the other P2X purinoceptors on the basis of its low affinity for purinoceptor antagonists such as suramin (5). The results obtained provide further insight into the binding properties of P2X purinoceptors and indicate that many, if not all, of the currently available purinoceptor antagonists may allosterically affect agonist binding to the P2X purinoceptor.

Experimental Procedures

Expression of the $P2X_4$ purinoceptor in CHO-K1 cells. The $P2X_4$ purinoceptor was expressed using the SFV expression system. The coding region of the rat $P2X_4$ purinoceptor was amplified by

ABBREVIATIONS: [^{35}S]ATPγS, [^{35}S]adenosine-5′-*O*-(3-thio)triphosphate; 2-Me-S-ATP, 2-methylthioadenosine triphosphate; αβ-MeADP, α,β-methylene ADP; αβ-MeATP, α,β-methylene ATP; βγ-MeATP β,γ-methylene ATP; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; P5P, pyridoxal-5′-phosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; SFV, Semliki forest virus; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

polymerase chain reaction and cloned into the *Bam*HI site of the pSFV1 vector and virus stocks produced as described previously (10–12). CHO-K1 cells were grown as a monolayer culture in 175-cm^2 flasks and infected with the virus stock at an approximate multiplicity of infection of 10. The cells were harvested 16 hr later in divalent cation-free phosphate-buffered saline and frozen on dry ice before preparation of membranes. Pulse-labeling experiments with [^{35}S]methionine confirmed that the P2X$_4$ purinoceptor was efficiently expressed (data not shown).

Receptor binding studies. The cell pellet from a 175-cm^2 flask was defrosted and homogenized using a Polytron tissue disrupter (full setting, 2 × 10-sec bursts) in 30 ml of an homogenizing buffer containing 50 mM Tris, 1 mM EDTA, 0.1% bacitracin, 0.02% soybean trypsin inhibitor, and 100 μM phenylmethylsulfonyl fluoride, pH 7.4 at 4°. The homogenate was centrifuged for 20 min at 48,000 × *g*, the supernatant was discarded, and the centrifuge tube and cell pellet were carefully rinsed with distilled water. The pellet was resuspended in homogenizing buffer using the Polytron tissue disrupter (setting 5 for 5 sec) and centrifuged as before. The pellet obtained was washed again by resuspension and centrifugation, then resuspended in 5 ml of homogenization buffer, and frozen at −85°. The frozen pellet was thawed, resuspended in 30 ml of homogenizing buffer using the Polytron tissue disrupter (setting 5 for 5 sec), and centrifuged for 20 min at 48,000 × *g*. This freeze/thaw cycle was repeated once, and the final pellet that was obtained was resuspended in an assay buffer of 50 mM Tris and 1 mM EDTA, pH 7.4 at 4°, and stored in aliquots at −85°. This extensive washing procedure was necessary to reduce contaminating ATP concentrations in the membrane preparation to <0.1 nM as assessed using the luciferin-luciferase technique (Sigma Chemical, St. Louis, MO).

Binding studies were carried out essentially as described previously (9, 10). All experiments were performed in assay buffers containing 1 mM EDTA to eliminate divalent cations because [^{35}S]ATPγS can be used only to label P2X purinoceptors in the absence of divalent cations (13). Studies were conducted at 4° to prevent nucleotide metabolism. Thus, under all of the ionic conditions used in this study, there was no detectable metabolism of 0.1 nM ATP.[1] In the majority of studies, the buffer also contained 50 mM Tris, although in some studies that were designed to examine the effect of ionic strength on [^{35}S]ATPγS binding, the 50 mM concentration of Tris was omitted and the buffer was supplemented with 5 mM HEPES and 5 mM *N*-methyl-D-glucamine (HEN buffer).

Reactions were always initiated by the addition of membranes. Incubations were performed at 4° for the indicated times in a final assay volume of 250 μl and were terminated by vacuum filtration over wet, 20 mM $Na_4P_2O_7$-pretreated, GF/B glass-fiber filters using either a Brandell 48-well or a Packard Filtermate cell harvester. The filters were washed for 20 sec with 10 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4 at 22°, and bound radioligand was determined by liquid scintillation spectrophotometry using either a Canbera Packard Topcount or 2200CA scintillation counter. Nonspecific binding of [^{35}S]ATPγS was defined using 10 μM ATPγS.

In the association, competition, and saturation studies, the reactions were performed in polystyrene 1-ml tube strips (Skatron, Cambridgeshire, UK). In the dissociation studies, the radioligand and membrane preparation were incubated for 120 min in a 50-ml polystyrene container (Sterilin, Staffordshire, UK), and dissociation was initiated by the addition of 250 μl of this pre-equilibrated radioligand/membrane preparation to the polystyrene 1-ml tube strips containing 100 μl of unlabeled ATPγS together with any other stated additions. In the kinetic and competition studies, the radioligand concentration was 0.1–0.2 nM, whereas in saturation studies, the radioligand concentration ranged from 0.01 to 2 nM. In all studies, total and nonspecific binding was determined in duplicate or, usually, triplicate. Unless otherwise stated, the data presented are the mean ± standard error of three to five separate experiments.

[1] A. D. Michel, unpublished observations.

Data analysis. Saturation binding data were analyzed using LIGAND with a modified *F* test to compare binding models (14). It was not possible to fit the data to models with the assumption of the presence of more than one population of specific binding site. However, in some experiments in which there was slight curvature in the Scatchard plot, the data were best described by the assumption of binding to a single population of saturable binding sites and to a second, nonspecific component of binding with capacity but no affinity. Using this two-component form of analysis resulted in a minor reduction (5–10%) in the estimate for B_{max} and a slight increase (10–20%) in the estimated K_D value compared with the parameter estimates obtained using the simple one-site analysis. Where appropriate, the parameters estimates from this two-component form of analysis were used when calculating mean values for B_{max} and K_D.

The competition binding data were analyzed using iterative curve-fitting procedures (15) to determine, initially, the pIC_{50} and Hill slope for the competition curve. If the Hill slope was less than unity, the data were fitted to a form of analysis in which it was assumed that the radioligand was labeling two populations of specific binding site. In this analysis, a pIC_{50} value for each site was calculated together with the proportion of the total specific binding that each site contained. To enable ready comparison with data from our previous studies, the IC_{50} values determined in this study were not adjusted to take into account the presence of radioligand and are presented as the negative logarithm of the IC_{50} (ie, pIC_{50}). Caveats regarding the calculation of equilibrium dissociation constants from such data have been discussed previously (10).

Kinetic data were analyzed using Prism (GraphPAD Software, San Diego, CA) with the assumption of association with, or dissociation from, either one or two populations of a specific binding site. In addition, the data were fitted to a model with the assumption of association with or dissociation from a single population of binding sites together with a very rapid component of either association or dissociation.

Materials. The sources of 2-Me-S-ATP, L-βγ-MeATP, ATPγS, αβ-MeATP, αβ-MeADP, ADP, ATP, βγ-MeATP, P5P, cibacron blue, DIDS, suramin, and PPADS were as described previously (10, 13). In addition, Coomassie blue (Coomassie brilliant blue G) and *d*-tubocurarine were purchased from Sigma Chemical (St. Louis, MO). The radioligand [^{35}S]ATPγS (specific activity, 1500 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK).

Results

Kinetic and saturation studies on [^{35}S]ATPγS binding to membranes prepared from P2X$_4$ purinoceptor-infected CHO-K1 cells. Preliminary kinetic studies were performed to identify conditions for the study of [^{35}S]ATPγS binding to membranes prepared from SFV/P2X$_4$ purinoceptor-infected CHO-K1 cells. In an assay buffer of 50 mM Tris and 1 mM EDTA at 4°, steady state levels of binding were achieved within 2–3 hr and remained stable for an additional ≥3 hr (Fig. 1). The kinetic data could be analyzed with the assumption of association with a single population of sites (K_{obs} = 0.024 ± 0.02 min^{-1}, $t_{1/2}$ = 29.9 ± 2.9 min, four experiments), although in two of the four experiments, a rapid initial component of association was also detected. Specific binding was readily reversible after the addition of 10 μM ATPγS. The dissociation data could be best described by assuming dissociation from a single population of sites (K_{-1} = 0.018 ± 0.001 min^{-1}, $t_{1/2}$ = 30.6 ± 4.8 min, four experiments). The kinetics of binding are described in more detail below.

In saturation studies, performed using a 3-hr incubation time, specific binding of [^{35}S]ATPγS represented 90–98% of total binding and increased with radioligand concentration

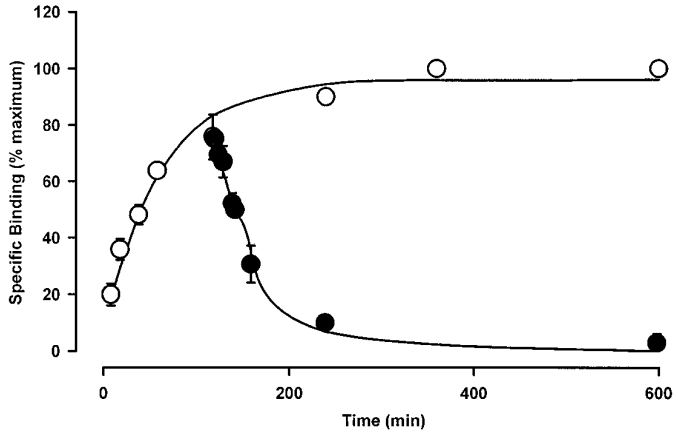

Fig. 1. Kinetics of [^{35}S]ATPγS binding to membranes prepared from CHO-K1 cells infected with the $P2X_4$ purinoceptor using a SFV construct. The data represent either the association with (○) or dissociation from (●) the $P2X_4$ purinoceptor of 0.1–0.2 nM [^{35}S]ATPγS. Dissociation was measured after 120 min of association by the addition of 10 μM ATPγS. In each case, values are mean ± standard error from four experiments. The data were normalized to the maximal level of binding measured after 360 min of association.

(Fig. 2). At the highest radioligand concentrations, specific binding seemed to saturate. Despite the slight curvature in the Scatchard plot of the specific binding data, which was evident at the highest radioligand concentrations, the data could only be analyzed with the assumption of binding of [^{35}S]ATPγS to a single population, rather than multiple populations, of specific binding sites. The radioligand K_D value was 0.13 nM ($pK_D = 9.9 \pm 0.03$), whereas the B_{max} value was 151 ± 8 pmol/mg of protein. This contrasts with the much lower levels of [^{35}S]ATPγS binding (K_D = 1.8 nM, B_{max} = 358 fmol/mg of protein) found in either noninfected CHO-K1 cells or CHO-K1 cells infected with the the *LacZ* gene as a control for SFV infection (9). Given the dramatic increase in [^{35}S]ATPγS binding in the cells infected with the $P2X_4$ purinoceptor, it is likely that [^{35}S]ATPγS was only labeling the $P2X_4$ purinoceptor in these cell membranes; therefore, for the remainder of the study, specific binding of [^{35}S]ATPγS to membranes prepared from the SFV-infected cells was considered to occur only to the $P2X_4$ purinoceptor.

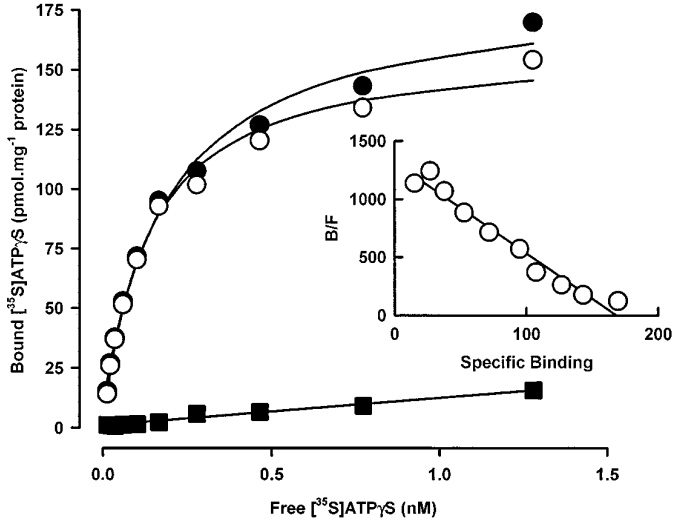

Fig. 2. Saturation binding of [^{35}S]ATPγS to $P2X_4$ purinoceptors. Total binding (●), specific binding (○), or nonspecific binding (■) was measured at radioligand concentrations of 0.02–1.4 nM. *Inset*, specific binding data are presented as a Scatchard plot. *Abscissa*, specific binding (pmol/mg of protein). *Ordinate*, ratio of specific binding to the free ligand concentration. The data are from a single experiment that was performed three times with similar results.

Competition studies on [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor. In competition studies, a number of nucleotide analogues competed for [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor. The Hill slopes for the competition curves were close to unity, with the exception of that for L-βγ-MeATP, and the data could only be described by assuming competition for a single population of noninteracting binding sites (Fig. 3a; Table 1).

In marked contrast, the competition curves obtained using a number of P2 purinoceptor antagonists were more complex (Fig. 3b). Cibacron blue and Coomassie blue were the most potent of the purinoceptor antagonists studied, producing significant inhibition of binding at low micromolar concentrations, although the competition curves obtained were clearly biphasic and the Hill slopes were less than unity. The competition curves for suramin, P5P, and PPADS were also characterized by a low Hill slope (Table 1), and these compounds only inhibited binding at concentrations of >10 μM. In the case of DIDS, the competition curve was also shallow,

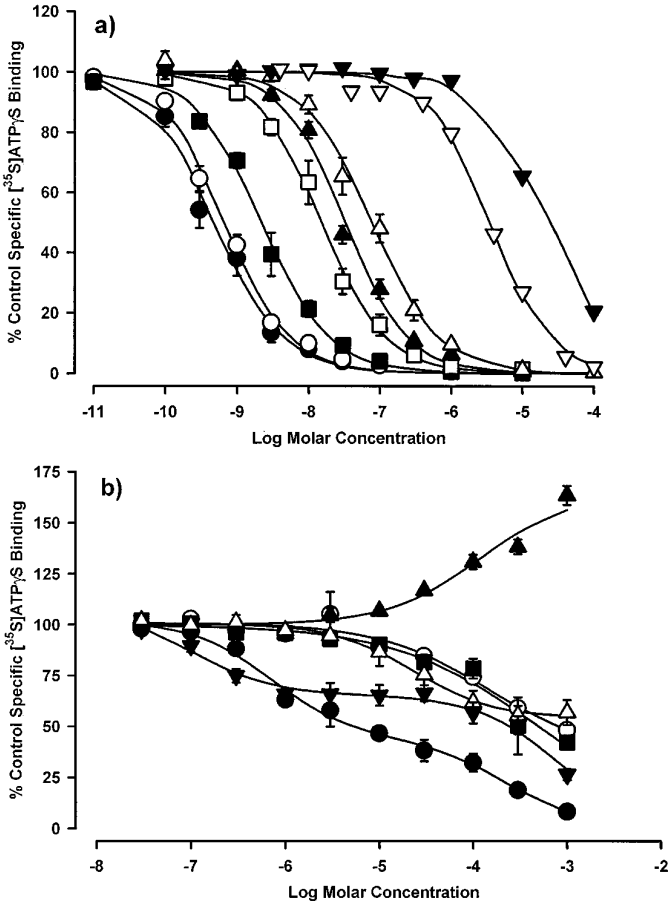

Fig. 3. Competition studies on $P2X_4$ purinoceptors labeled with 0.1–0.2 nM [^{35}S]ATPγS. a, Competition by ATP (●), ATPγS (○), 2-Me-S-ATP (■), αβ-MeATP (□), ADP (▲), βγ-MeATP (△), L-βγ-MeATP (▽), and αβ-MeADP (▼). b, Competition by cibacron blue (●), Coomassie blue (▼), suramin (○), DIDS (△), PPADS (■), and *d*-tubocurarine (▲). Values are mean ± standard error of three determinations.

TABLE 1

Competition studies on the rat recombinant $P2X_4$ purinoceptor

Competition binding studies were performed in membranes prepared from CHO-K1 cells infected with the rat $P2X_4$ purinoceptor using a Semliki Forest virus construct. The concentration of [^{35}S]ATPγS was 0.1–0.2 nM. Data are mean ± standard error of three to five determinations.

Compound	pIC_{50}	Hill slope
ATP	9.32 ± 0.12	0.92 ± 0.03
ATPγS	9.17 ± 0.06	0.96 ± 0.04
2-Me-S-ATP	8.66 ± 0.09	0.94 ± 0.05
αβ-MeATP	7.82 ± 0.11	0.99 ± 0.02
ADP	7.48 ± 0.05	1.01 ± 0.04
βγ-MeATP	7.11 ± 0.10	0.95 ± 0.06
L-βγ-MeATP	5.71 ± 0.16	0.84 ± 0.01
αβMeADP	4.66 ± 0.16	0.97 ± 0.03
Dextran[a]	0.88 ± 1.60	0.15 ± 0.04
DIDS[a]	1.10 ± 0.28	0.41 ± 0.03
PPADS	3.33 ± 0.18	0.26 ± 0.08
P5P	2.89 ± 0.10	1.35 ± 0.35
Cibacron blue	5.03 ± 0.17	0.46 ± 0.03
Suramin	3.27 ± 0.14	0.50 ± 0.02
Coomassie blue	3.93 ± 0.30	0.25 ± 0.00

[a] pIC_{50} value estimated is in excess of the highest concentration of the compound tested.

and it seemed that this compound only inhibited a proportion of [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor. The nicotinic receptor antagonist *d*-tubocurarine potentiated binding at concentrations of >10 μM.

Given the complex nature of the competition curves, cibacron blue and *d*-tubocurarine were examined for their effect on the saturation binding properties of [^{35}S]ATPγS. Cibacron blue (10 μM) decreased the B_{max} value from 151 ± 8 to 84 ± 2 pmol/mg of protein, whereas *d*-tubocurarine (300 μM) increased the B_{max} value (174 ± 7 pmol/mg of protein). Neither *d*-tubocurarine nor cibacron blue affected the radioligand K_D value. Thus, pK_D values in the absence or presence or either 10 μM cibacron blue or 300 μM *d*-tubocurarine were 9.9 ± 0.03, 9.8 ± 0.04, and 9.9 ± 0.02, respectively.

Effects of purinoceptor antagonists of [^{35}S]ATPγS dissociation kinetics. The ability of the antagonists to change the radioligand B_{max} value without affecting its affinity, together with the complex inhibition curves produced in competition studies, suggested that the antagonists could be affecting [^{35}S]ATPγS binding in an allosteric manner. To examine this further, their affect on the dissociation kinetics of the radioligand was examined.

Dissociation of [^{35}S]ATPγS from the $P2X_4$ purinoceptor was measured by isotopic dilution with 10 μM ATPγS of a pre-equilibrated radioligand/$P2X_4$ purinoceptor preparation in the either presence or absence of purinoceptor antagonists. The level of [^{35}S]ATPγS binding measured 25 min after initiation of dissociation was found to be increased by *d*-tubocurarine but decreased by suramin and cibacron blue (Fig. 4a). These effects of the purinoceptor antagonists were concentration dependent; approximate pEC_{50} values for the ability of cibacron blue, *d*-tubocurarine, and suramin to modify radioligand dissociation were 5.8 ± 0.1, 4.2 ± 0.1, and 3.6 ± 0.2, respectively.

The effect of a number of other purinoceptor antagonists on [^{35}S]ATPγS dissociation from the $P2X_4$ purinoceptor were examined at a single concentration of antagonist that produced a ~50% inhibition of binding in competition studies (Fig. 4b). DIDS, Coomassie blue, PPADS, and P5P increased dissociation of [^{35}S]ATPγS, whereas the nicotinic receptor

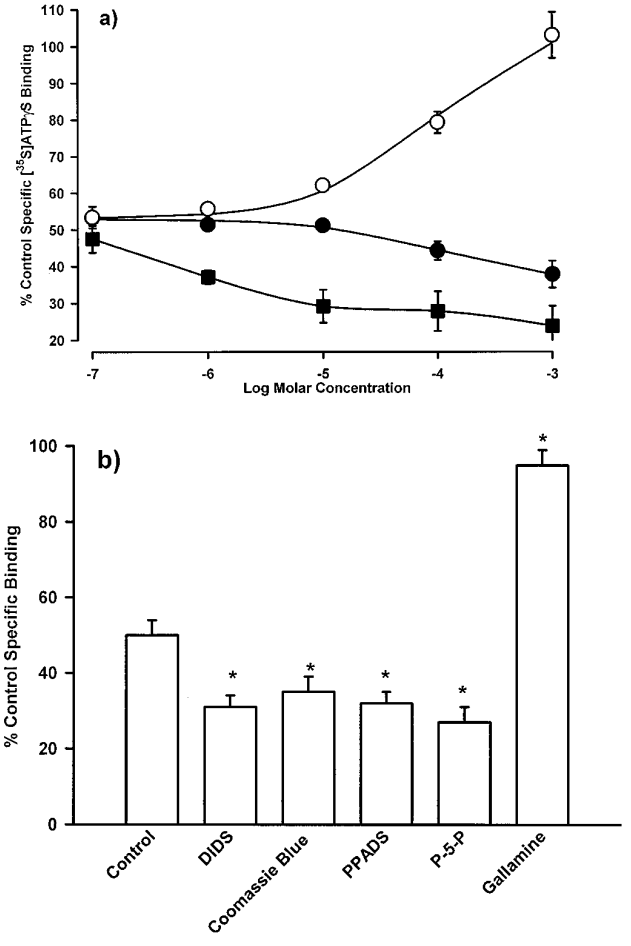

Fig. 4. Effect of purinoceptor antagonists on [^{35}S]ATPγS dissociation from the $P2X_4$ purinoceptor. Specific binding of 0.1–0.2 nM [^{35}S]ATPγS was measured 25 min after initiation of dissociation by the addition of a pre-equilibrated radioligand/$P2X_4$ purinoceptor mixture to 10 μM ATPγS either with or without antagonists. a, Dissociation was studied in the presence or absence of the indicated concentrations of cibacron blue (■), suramin (●), or *d*-tubocurarine (○). In the absence of antagonists, [^{35}S]ATPγS binding measured 25 min after initiation of dissociation was 50 ± 1% of binding measured before initiation of dissociation. b, Dissociation was studied in the absence (*control*) or presence of 1 mM DIDS, 10 μM Coomassie blue, 0.1 mM PPADS, 1 mM P5P, or 0.1 mM gallamine. Values are mean ± standard error of three determinations. In each experiment, the specific binding data have been expressed as a percentage of the control level of [^{35}S]ATPγS binding measured before initiation of dissociation. *, $p < 0.05$, significantly different than the level of [^{35}S]ATPγS binding measured in the control dissociation group (analysis of variance and Dunnett's *t* test).

antagonist gallamine reduced the apparent dissociation of [^{35}S]ATPγS. ATP, αβ-MeATP, and ADP at concentrations of 1 nM, 100 nM, and 10 μM, respectively, did not affect the dissociation of [^{35}S]ATPγS induced by 10 μM ATPγS (data not shown).

The effect of a single concentration of either *d*-tubocurarine (1 mM) or cibacron blue (10 μM) on the rate of [^{35}S]ATPγS dissociation from the $P2X_4$ purinoceptor was examined (Fig. 5). From this more detailed kinetic study, it can be seen that the main effect of *d*-tubocurarine in dissociation studies was to produce an initial increase in the measured level of [^{35}S]ATPγS binding. This effect represented a 115 ± 37% increase in [^{35}S]ATPγS binding compared with that measured before initiation of dissociation and was evident at the

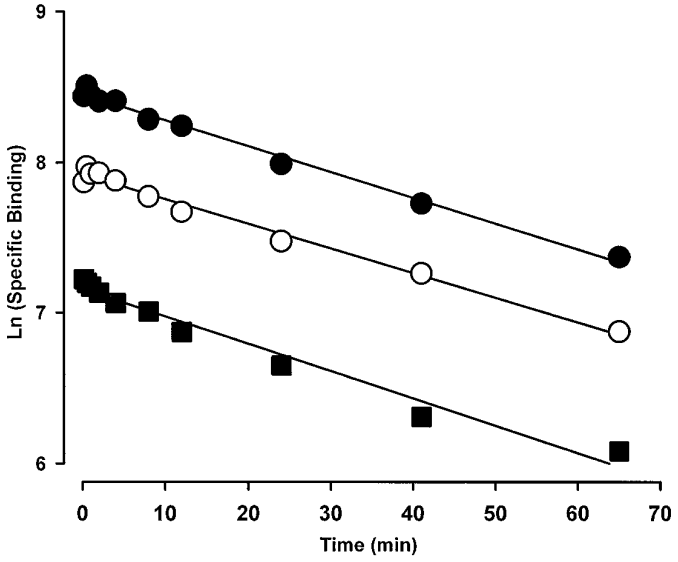

Fig. 5. Effect of cibacron blue and *d*-tubocurarine on the kinetics of [^{35}S]ATPγS dissociation from the P2X$_4$ purinoceptor. Specific binding of [^{35}S]ATPγS was measured after initiation of dissociation by the addition of 10 μM ATPγS to a pre-equilibrated radioligand/P2X$_4$ purinoceptor mixture. Dissociation was studied in the absence (○) or presence of 10 μM cibacron blue (■) or 1 mM *d*-tubocurarine (●). The figure represents a semilogarithmic transform in which the slope of the plot is proportional to the rate constant for dissociation. Each curve originated from the same predissociation level of binding, which in this experiment was 2870 dpm (ln 7.96). At the first time point shown (10 sec), both *d*-tubocurarine and cibacron blue produced large changes in the level of binding that was detected. Values are from a single experiment that was performed three times with similar results.

first time point studied (10 sec). Thereafter, the rate of dissociation of the radioligand measured in the presence of *d*-tubocurarine (K_{-1} = 0.017 ± 0.001 min^{-1}, determined from an analysis of all time points of >1 min) was similar to that measured in the absence of *d*-tubocurarine (K_{-1} = 0.018 ± 0.001 min^{-1}).

In the presence of 10 μM cibacron blue, dissociation of [^{35}S]ATPγS was biphasic. Thus, within 5 min of initiation of dissociation in the presence of 10 μM cibacron blue, there was a 45 ± 8% reduction in [^{35}S]ATPγS binding compared with that measured before initiation of dissociation. Thereafter, the rate of radioligand dissociation in the presence of cibacron blue (K_{-1} = 0.031 ± 0.007 min^{-1}, determined from an analysis of all time points of >5 min) was not much different from that measured in its absence (K_{-1} = 0.018 ± 0.001 min^{-1}).

Effect of monovalent cations on [^{35}S]ATPγS binding. In preliminary studies, it was noted that monovalent cations exhibited marked effects on [^{35}S]ATPγS binding. Because *d*-tubocurarine is cationic, studies were undertaken to investigate whether the monovalent cations also affected [^{35}S]ATPγS binding. Because the Tris cation can mimic the effects of monovalent cations, these experiments were performed in a buffer of 5 mM HEPES and 1 mM EDTA in which pH was adjusted to 7.4 by the addition of 5 mM *N*-methyl-D-glucamine (HEN buffer). The effects of the monovalent cations were very pronounced, with short incubation periods; to assess the structure-activity relationship, their effects were studied using a 5-min association period. All monovalent cations studied were able to increase [^{35}S]ATPγS binding, with EC$_{50}$ values for methylguanidinium, sodium, lithium, potassium, and choline of 36 ± 6, 29 ± 3, 28 ± 4, 29 ± 4, and 33 ± 4 mM, respectively (Fig. 6a). Although guanidinium was of similar potency, an EC$_{50}$ value cannot be given because no clear maximum was observed. *d*-Tubocurarine (Fig. 6b) also potentiated binding to a similar extent but with much higher potency (EC$_{50}$. = 0.28 ± 0.13 mM). Sucrose at concentrations of 1–300 mM did not affect [^{35}S]ATPγS binding (Fig. 6a).

The effects of *d*-tubocurarine and the monovalent cation sodium were not additive. Thus, the maximal potentiation of [^{35}S]ATPγS binding produced by NaCl and *d*-tubocurarine were similar, and in the presence of a maximally effective concentration of NaCl, *d*-tubocurarine did not further potentiate binding (Fig. 6b).

Effects of sodium and *d*-tubocurarine on association kinetics and saturation binding properties of [^{35}S]ATPγS. The influence of NaCl (150 mM) and *d*-tubocurarine (1 mM) on the association kinetics and saturation binding properties of [^{35}S]ATPγS were examined further to determine how these agents increased radioligand binding. In the low-ionic-strength HEN buffer, [^{35}S]ATPγS binding

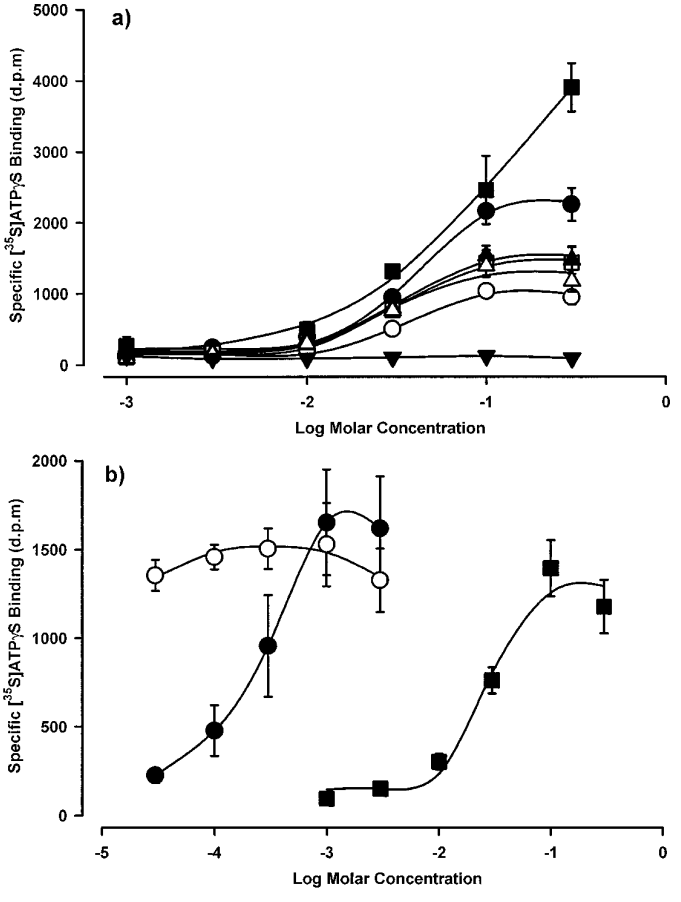

Fig. 6. Ability of monovalent cations to increase [^{35}S]ATPγS binding to the P2X$_4$ purinoceptor. [^{35}S]ATPγS binding to the P2X$_4$ purinoceptor was determined after a 5-min incubation in a buffer of 5 mM HEPES and 5 mM *N*-methyl-D-glucamine HCl. a, Incubations were performed in the presence of the indicated concentrations of methyl-guanidinium (●), guanidinium (■), choline (○), potassium (□), lithium (▲), sodium (△), or sucrose (▼). b, Incubations were performed in the presence of the indicated concentrations of NaCl (■), *d*-tubocurarine (●), or *d*-tubocurarine in the presence of 150 mM NaCl (○). Values are mean ± standard error of three determinations. In the absence of added cations or *d*-tubocurarine, specific binding was 150 dpm. All monovalent cations were added as their chloride salts.

could be described with the assumption of a simple, bimolecular, reversible association of [^{35}S]ATPγS with the P2X$_4$ purinoceptor. The rate of association was very slow (K_{obs} = 0.006 ± 0.001 min^{-1}, $t_{1/2}$ = 112 min), and the equilibrium level of binding was lower than that achieved in HEN buffer containing either 150 mM NaCl or 1 mM *d*-tubocurarine (Fig. 7). Thus, steady state levels of binding were only 30 ± 12% of those determined in the presence of 1 mM *d*-tubocurarine. In HEN buffer containing either 1 mM *d*-tubocurarine or 150 mM NaCl, the association of the radioligand was much faster, and the data could be described by assuming two components of [^{35}S]ATPγS binding. There was an initial very rapid phase of association, which seemed to be complete at the first time point measured (10 sec). This made it impossible to determine the kinetic parameters for this component using a filtration binding assay, although it was possible to estimate its magnitude by fitting the data to a model in which binding was assumed to start from a "nonzero" level. Using this approach, the rapid component of [^{35}S]ATPγS association in the presence of 1 mM *d*-tubocurarine was estimated to represent 16 ± 3% of the steady state level of binding, whereas in the presence of 150 mM NaCl, the rapid component represented 22 ± 10% of the steady state level of [^{35}S]ATPγS binding. The remainder of the association data could be accounted for by assuming association of [^{35}S]ATPγS with a single population of binding sites. The observed rates of association for this slower component of binding measured in the presence of 1 mM *d*-tubocurarine (K_{obs} = 0.054 ± 0.014 min^{-1}, $t_{1/2}$ = 14.7 ± 3.8 min) or 150 mM NaCl (K_{obs} = 0.054 ± 0.016 min^{-1}, $t_{1/2}$ = 15.2 ± 3.8 min) were similar.

In saturation studies conducted using HEN buffer (Fig. 8), the B_{max} values determined in the presence of either 1 mM *d*-tubocurarine (143 ± 11 pmol/mg of protein) or 150 mM NaCl (113 ± 5 pmol/mg of protein) were greater than those measured in their absence (54 ± 4 pmol/mg of protein). Similarly, the K_D values determined in the presence of either 1 mM *d*-tubocurarine (pK_D = 10.28 ± 0.04, K_D = 0.052 nM) or 150 mM NaCl (pK_D = 10.07 ± 0.02, K_D = 0.085 nM) were greater than the K_D value measured in their absence (pK_D = 9.56 ± 0.06, K_D = 0.276 nM). The effect of *d*-tubocurarine in the low-ionic-strength buffer was more marked than that seen in 50 mM Tris buffer (see below).

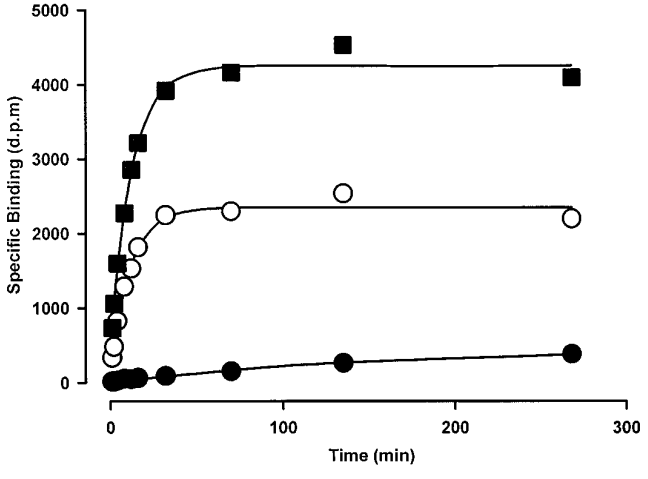

Fig. 7. Effect of *d*-tubocurarine and NaCl on the kinetics of [^{35}S]ATPγS association with the P2X$_4$ purinoceptor. Specific binding of 0.1–0.2 nM [^{35}S]ATPγS to the P2X$_4$ purinoceptor was determined in a buffer of 5 mM HEPES and 5 mM *N*-methyl-D-glucamine HCl in the absence (●) or the presence of 150 mM NaCl (○) or 1 mM *d*-tubocurarine (■). Values are from a single experiment that was performed three times with similar results.

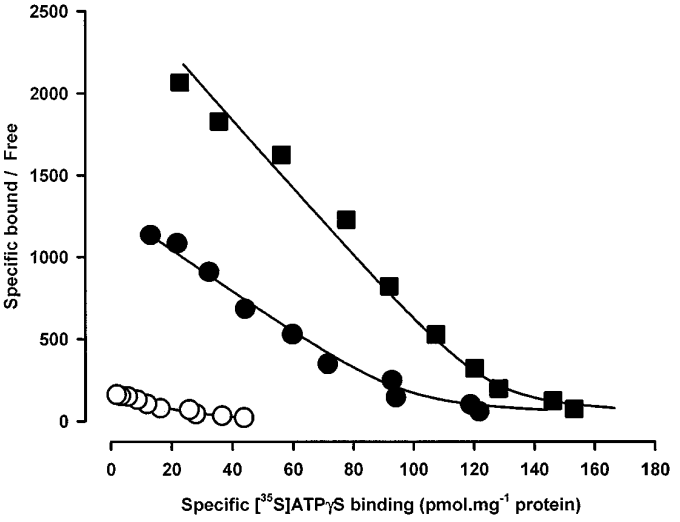

Fig. 8. Scatchard plot of the effect of *d*-tubocurarine and NaCl on saturation binding of [^{35}S]ATPγS to the P2X$_4$ purinoceptor. Specific binding of 0.02–1.4 nM [^{35}S]ATPγS to the P2X$_4$ purinoceptor was determined in a buffer of 5 mM HEPES and 5 mM *N*-methyl-D-glucamine HCl in the absence (○) or the presence of either 150 mM NaCl (●) or 1 mM *d*-tubocurarine (■). Values are from a single experiment that was performed three times with similar results.

Discussion

The main finding of the current study was that the rat P2X$_4$ purinoceptor could be labeled using [^{35}S]ATPγS and that purinoceptor antagonists and monovalent cations could modulate the binding of the radioligand to the P2X$_4$ purinoceptor.

In saturation and competition studies, [^{35}S]ATPγS bound with very high affinity to a high density of sites in CHO-K1 cells expressing the P2X$_4$ purinoceptor using the SFV infection system. Although CHO-K1 cells express endogenous [^{35}S]ATPγS binding sites, these sites are present at a relatively low density (compared with the P2X$_4$ purinoceptors), and their binding characteristics are very different than those determined in the P2X$_4$ purinoceptor-expressing cells (9). Consequently, we are confident that any [^{35}S]ATPγS binding detected in the SFV-P2X$_4$ purinoceptor-infected CHO-K1 cells reflects labeling of the recombinant P2X$_4$ purinoceptor. Interestingly, the level of expression of the P2X$_4$ purinoceptor achieved using SFV was remarkably high. Thus, even though high-level expression of the P2X$_1$, P2X$_2$, and P2X$_3$ purinoceptors was achieved using SFV (B_{max} = 2–40 pmol/mg of protein), the B_{max} value for the P2X$_4$ purinoceptor was even higher (151 pmol/mg of protein). The reason for the high level of expression of the P2X$_4$ purinoceptor is not known, but this result illustrates the effectiveness of the SFV expression system in achieving high levels of receptor expression.

In competition studies, all nucleotide ligands that were examined competed with [^{35}S]ATPγS for binding to the P2X$_4$ purinoceptor in an apparently simple competitive manner. This is similar to results obtained using the recombinant P2X$_1$, P2X$_2$, and P2X$_3$ purinoceptors, which have also been

studied using [^{35}S]ATPγS as radioligand (9, 10, 16). The affinity estimates for the agonists were similar to those determined at the $P2X_1$, $P2X_2$, and $P2X_3$ purinoceptors, and few of the nucleotides exhibited any marked selectivity (9, 10, 16). The only exceptions were 2-Me-S-ATP and αβ-MeATP, which possess slightly higher affinity for the $P2X_3$ purinoceptor (16) than for the other P2X purinoceptors.

In contrast to the results obtained using the agonists, the antagonists possessed lower potency at inhibition of [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor than at inhibition of binding to the $P2X_1$, $P2X_2$, and $P2X_3$ purinoceptors. This was particularly evident in the case of suramin, which possessed almost 1000-fold lower affinity at the $P2X_4$ purinoceptor than at the $P2X_1$, $P2X_2$, or $P2X_3$ purinoceptor. These findings are in keeping with functional studies that have highlighted the antagonist insensitivity of the $P2X_4$ purinoceptor (5, 6).

In binding studies on the $P2X_1$, $P2X_2$, and $P2X_3$ purinoceptors, most antagonists produced competition curves with Hill slopes close to unity, suggesting a competitive interaction with the P2X purinoceptor (9, 10, 16). The unexpected finding in the current study was that all of the antagonists produced complex inhibition curves at the $P2X_4$ purinoceptor. Cibacron blue and Coomassie blue produced biphasic competition curves, whereas suramin, PPADS, P5P, and DIDS inhibited [^{35}S]ATPγS binding only at high concentrations, and the Hill slopes for the competition curves were less than unity. The most striking result was obtained with *d*-tubocurarine, which potentiated binding in a concentration-dependent manner. This nicotinic receptor antagonist has been shown to antagonize cellular responses mediated by $P2X_2$ purinoceptors at similar concentrations to those that affected binding (2, 17).

This complex action of the antagonists may reflect allosteric interactions of the compounds with the $P2X_4$ purinoceptor because in saturation studies in Tris buffer, cibacron blue and *d*-tubocurarine changed the radioligand B_{max} value with little effect on the K_D value. Furthermore, in kinetic experiments, the compounds differentially affected dissociation of the radioligand. However, definitive evidence for an allosteric mechanism awaits further study. Indeed, if the effects are allosteric, they are atypical. Thus, in studies on other receptor systems such as the muscarinic receptor, allosteric regulators have been shown to mainly affect the dissociation rate of the radioligand (18, 19). In contrast, at the $P2X_4$ purinoceptor, the main effect of *d*-tubocurarine and cibacron blue in dissociation studies was to produce a very rapid (10 sec to 5 min) increase or decrease, respectively, in the level of [^{35}S]ATPγS binding detected in the filtration binding assay. The increase in binding produced by *d*-tubocurarine was very rapid in onset, being complete within 10 sec, whereas the decrease in binding produced by cibacron blue was somewhat slower (1–5 min for completion). After the initial rapid change in the level of [^{35}S]ATPγS binding detected, the radioligand dissociation rate did not seem to be significantly affected by *d*-tubocurarine and was only slightly increased by cibacron blue.

In addition to the purinoceptor antagonists, monovalent cations could also modulate [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor. Thus, in a low-ionic-strength buffer, the kinetics of [^{35}S]ATPγS binding were relatively slow, the radioligand possessed low affinity, and the steady state level of binding was reduced compared with that measured in a 50 mM Tris/1 mM EDTA buffer. Under these conditions, [^{35}S]ATPγS binding could be increased by a number of monovalent cations. The effects of the ions were not secondary to changes in osmolarity because sucrose did not mimic the effects of the ions. There did not seem to be any ionic specificity for these effects because all monovalent cations increased [^{35}S]ATPγS binding with similar potency, although the maximal increases in binding produced by methyl-guanidinium and guanidinium were slightly greater than those produced by the other ions.

The increase in [^{35}S]ATPγS binding produced by the monovalent cation sodium was associated with an increase in the observed rate of radioligand association, the radioligand K_D value, and the B_{max} value. Interestingly, in low-ionic-strength buffer, *d*-tubocurarine produced similar effects on [^{35}S]ATPγS binding, and because the effects of *d*-tubocurarine and NaCl did not seem to be additive, it is possible that both agents interact at a common site on the $P2X_4$ purinoceptor to affect [^{35}S]ATPγS binding. The actions of cibacron blue in kinetic dissociation studies were the converse of those produced by *d*-tubocurarine, and it would have been interesting to examine the interaction of these agents in more detail. However, this was not possible because the solubility of cibacron blue was reduced in the presence of *d*-tubocurarine.[2]

Although these results are indicative of allosteric effects of monovalent cations and purinoceptor antagonists on [^{35}S]ATPγS binding, it is not possible at the present to suggest a definitive mechanistic model to describe the overall system. However, given the high affinity of ATP for the $P2X_4$ purinoceptor in binding studies (IC_{50} < 1 nM) compared with its potency (EC_{50} = 10 μM) in functional studies (5, 6), it seems most unlikely that purinoceptor agonists are labeling a functional state of the receptor. Instead, it is possible that purinoceptor agonists label a high affinity, desensitized state of the P2X purinoceptor as we have previously suggested (9, 10, 20). In this case, binding might be described in terms of a conceptual model that includes one or more receptor isomerization steps such that R + L ⇆ RL ⇆ DL, in which in its simplest form R and L refer to the receptor and ligand, respectively, and D represents a higher affinity, desensitized state of the receptor. In binding studies, it is probable that only D, the high affinity state of the receptor, would be detected using a filtration binding assay. One might further assume that there is one or more populations of allosteric sites on the receptor responsible for the positive and negative effects produced by sodium, *d*-tubocurarine, and the purinoceptor antagonists. The rapid increase in binding, together with no change in subsequent radioligand dissociation rate, produced by *d*-tubocurarine (and presumably sodium ions) would be consistent with these agents favoring conversion of the receptor into the high affinity state (DL) by increasing the rate constant for conversion of RL to DL. Under such circumstances, it is possible that during dissociation experiments conducted in the presence of *d*-tubocurarine, there was a rapid conversion of pre-existing RL into DL, leading to the apparent increase in binding. Conversely, the purinoceptor antagonists might bind at the same or a different site to favor the conversion of DL to RL, the low affinity state, presumably

[2] A. D. Michel and K. J. Miller, unpublished observations.

in addition to their ability to compete for a common site with ATP. Such an action would then lead to a reduction in the measured level of binding.

Relating these allosteric effects seen in binding experiments to data from functional studies is difficult for several reasons. First, our studies were carried out at 4°, but we have evidence that the phenomena described are also evident at room temperature,[3] and therefore they presumably are functionally relevant. Second, the $P2X_4$ purinoceptor gene has only recently been cloned, and the receptor pharmacology has not been extensively studied. However, the demonstration (5) that a lysine present in the $P2X_1$ and $P2X_2$ purinoceptors but absent in the $P2X_4$ purinoceptor is critical for the binding of antagonists but not agonists would be consistent with the ability of antagonists to bind to the $P2X_4$ purinoceptor at a site distinct from, or additional to, that at which ATP binds. In functional studies on the $P2X_4$ purinoceptor, PPADS (5) and cibacron blue (6) have been shown to potentiate responses to ATP, and it is possible that these effects may be related to their allosteric properties. Thus, if [^{35}S]ATPγS does indeed label a high affinity desensitized state or states of the $P2X_4$ purinoceptor, then the finding that cibacron blue and PPADS reverse or inhibit formation of this desensitized state suggests that these compounds may prevent or reverse receptor desensitization, thereby increasing the available pool of receptors capable of activation in functional studies. Because $P2X_4$ purinoceptor-mediated responses are known to desensitize in the continued presence of agonist (6), such an action could potentiate ATP-mediated responses.

The putative ability to prevent or reverse receptor desensitization may also account for previous reports of purinoceptor antagonists potentiating P2X purinoceptor-mediated responses. Thus, in human bladder, low concentrations of Coomassie blue potentiate αβ-MeATP-induced contractions (21), whereas in rat vas deferens, cibacron blue (22), Evans blue (23), and suramin (24) can increase the maximal tissue response to this metabolically stable P2X purinoceptor agonist, in addition to antagonizing αβ-MeATP-induced contractions. Similarly, suramin has been shown to potentiate rather than inhibit ATP-activated conductances in guinea pig myenteric neurons (25), while in rat vagus nerve, both suramin and cibacron blue can potentiate the maximal depolarizing responses to αβ-MeATP (26). Conversely, if the increased binding of [^{35}S]ATPγS in the presence of *d*-tubocurarine is due to its ability to convert the P2X purinoceptor into a desensitized state, then it is possible that *d*-tubocurarine may be able to block the P2X purinoceptor by promoting receptor desensitization. In this respect, ATP-activated inward currents in PC12 cells desensitize in the presence but not in the absence of *d*-tubocurarine (17).

In conclusion, this study has provided additional evidence that [^{35}S]ATPγS can be used to label recombinant P2X purinoceptor types. The binding and functional data show some agreement in that a number of reported purinoceptor antagonists possess low affinity in binding to the $P2X_4$ purinoceptor in both functional and binding studies. However, the interaction of the antagonists with the $P2X_4$ purinoceptor is clearly complex and may involve an allosteric interaction that may explain, at least in part, the potentiating effects of the specific P2 purinoceptor antagonists described in functional studies. Our data are in keeping with the concept that the P2X purinoceptor will be similar to other ligand-gated cation channels, such as the γ-aminobutyric acid type A and nicotinic receptors, in possessing multiple sites for modulation of receptor function.

[3] A. D. Michel and K. J. Miller, unpublished observations.

Acknowledgments

We thank Danielle Estoppey and Yves Humbert for their help in preparing SFV stocks and infecting CHO-K1 cells. In addition, we are grateful to Danielle Estoppey for testing the SFV-infected CHO-K1 cells for functional receptor expression using electrophysiology.

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