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G Protein-Dependent Activation of Phospholipase C by Adenosine A_3 Receptors in Rat Brain

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SUMMARY

The recently cloned G protein-coupled adenosine A_3 receptor has been proposed to play a role in the pathophysiology of cerebral ischemia. Because phospholipase C activation occurs as a very early response to brain ischemia, we evaluated the ability of A_3-selective and nonselective adenosine analogues to elicit phosphoinositide hydrolysis. In *myo*-[^{3}H]inositol-labeled rat striatal and hippocampal slices, A_3 agonists stimulated formation of [^{3}H]inositol phosphates in a concentration-dependent manner. In striatum, the potency order was 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide ≥ N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide ≫ *N*-methyl-1,3-di-n-butylxanthine-7-β-D-ribofuronamide ≥ 5′-*N*-ethylcarboxamidoadenosine ≥ N^6-2-(4-aminophenyl)-ethyladenosine > N^6-(*p*-sulfophenyl)-adenosine = 1,3-dibutylxanthine-7-riboside, which is identical to the potency order in binding studies at cloned rat A_3 receptors. Stimulation of phospholipase C activity was abolished by guanosine-5′-*O*-(2-thiodiphosphate), confirming the involvement of a G protein-coupled receptor. Activation of phospholipase C was higher in the striatum than in the hippocampus, consistent with A_3 receptor densities. Stimulation of phospholipase C activity by adenosine analogues was only modestly antagonized by xanthine derivatives and at much higher concentrations than needed for blocking adenosine A_1, A_{2A}, and A_{2B} receptors. In the presence of an A_1/A_2 antagonist, a selective A_3 agonist only weakly inhibited forskolin-stimulated adenylyl cyclase activity in rat striatum. Thus, stimulation of phospholipase C activity represents a principal transduction mechanism for A_3 receptors in mammalian brain, and perhaps A_3 receptor-mediated increases of inositol phosphates in the ischemic brain contribute to neurodegeneration by raising intracellular calcium levels.

Four G protein-coupled adenosine receptors have been cloned and designated A_1, A_{2A}, A_{2B}, and A_3 subtypes (1). These receptors share features common to other members of the G protein-coupled receptor superfamily, including seven hydrophobic putative transmembrane domains linked by regions of greater hydrophilicity (1–3). The first three of these receptors were identified initially on the basis of physiological and pharmacological criteria. However, the A_3 receptor was defined only after its recent molecular cloning. The rat A_3 sequence, tgpcr1, was first cloned by Meyerhof *et al.* (4) from a rat testis cDNA library by a polymerase chain reaction-based strategy. Subsequently, the functional expression of a rat striatal cDNA encoding the identical receptor confirmed the sequence as an adenosine receptor based on agonist-induced inhibition of adenylyl cyclase activity (5) and specific binding of the radioligand [^{125}I]APNEA. More recently, sheep (6) and human (7, 8) A_3 receptors have been cloned. Rat A_3 receptors are relatively insensitive to xanthines, known to be potent A_1 and A_{2A} receptor antagonists (5, 9). However, there are major species differences in the pharmacological profile of A_3 receptors with respect to xanthines (10, 11).

The physiological role of the A_3 receptor is mostly unex-

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ABBREVIATIONS: [^{125}I]APNEA, N^6-2-(4-amino-3-iodophenyl)-ethyladenosine; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methyuronamide adenosine; CHO, Chinese hamster ovary; PLC, phospholipase C; InsP, inositol phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; DBXRM, *N*-methyl-1,3-di-*n*-butylxanthine-7-β-D-ribofuronamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; APNEA, N^6-2-(4-aminophenyl)-ethyladenosine; SPA, N^6-(*p*-sulfophenyl)-adenosine; DBXR, 1,3-dibutylxanthine-7-riboside; CPX, 8-cyclopentyl-1,3-dipropylxanthine; XAC, 8-[4-[[[[(2-aminoethyl)amino-]carbonyl]methyl]-oxy]phenyl]-1,3-dipropylxanthine; BWA1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; KHB, Krebs-Henseleit buffer; ADA, adenosine deaminase; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); [^{125}I]AB-MECA, N^6-(4-amino-3-iodobenzyl)-adenosine-5′-*N*-methyluronamide.

plored. A_3 receptor transcripts have been found in testes (4–7), heart (5, 8), lung (5–8), immune system (12), and various brain areas (5–8). A variety of adenosine-elicited responses have been characterized as xanthine insensitive, thus suggesting involvement of A_3 receptors in hypotension (13), ischemic preconditioning of the heart (14), and release of inflammatory mediators from mast cells (12, 15). However, a precise characterization of the physiological role of the A_3 receptor has been hampered by the lack of selective A_3 receptor ligands.

The first truly selective A_3 receptor agonists to be synthesized were the N^6-benzyladenosine-5′-uronamides (16–18). IB-MECA was shown to display a 50-fold selectivity for rat brain A_3 versus either A_1 or A_{2A} receptors in binding studies using CHO cells transfected with the cloned A_3 receptor (16). More recently, 2-substitution of N^6-benzyl-adenosine-5′-uronamides was shown to further enhance A_3 receptor selectivity, leading to Cl-IB-MECA. The latter compound is selective for A_3 versus A_1 and A_{2A} receptors by 2500- and 1400-fold, respectively (17).

Insights into the role(s) of A_3 receptors in brain have come from recent *in vivo* studies using IB-MECA. In mice, administration of this compound produced a locomotor depressant response that was insensitive to A_1 or A_{2A} receptor-selective xanthine antagonists (18). The chronic administration of IB-MECA was found to be cerebroprotective against *N*-methyl-D-aspartate-induced seizures in mice (19) and against ischemia-associated neurodegeneration in gerbils (20). Opposite effects, i.e., enhanced mortality and extensive neuronal destruction in the hippocampus of ischemic animals, were induced by acute IB-MECA administration (20). These data have led to the hypothesis that activation of brain A_3 receptors may play an important role in the development of ischemic damage (20) and that desensitization of this receptor after chronic agonist administration may prove to be useful in counteracting ischemia-associated brain damage.

Very little is known about transduction mechanisms involved in A_3 receptor effects *in vivo*. In cell culture, two second messenger systems have been found to be coupled to A_3 receptors: inhibition of adenylyl cyclase and activation of PLC. Potencies of adenosine agonists in inhibiting adenylyl cyclase via A_3 receptors correlated with their relative binding affinities (9, 17). In RBL-2H3 mast cells (15, 21), activation of a xanthine-insensitive adenosine receptor resulted in stimulation of PLC and formation of inositol-1,4,5-triphosphate. cDNA-hybridization studies confirmed the presence of the A_3 subtype in this cell line (12); however, the agonist order of potency in this response does not match that predicted for A_3 receptors.[1] Moreover, RBL-2H3 mast cells and other mast cells also express other adenosine receptor subtypes (21, 22), which makes interpretation of functional responses complex.

In the brain, PLC activation and subsequent formation of InsPs and diacylglycerol might be relevant to ischemic damage because this transduction pathway has been proposed to represent a very early pathophysiological response to cerebral ischemia (23–26). All previous studies (27, 28) have failed to demonstrate either direct activation or inhibition of basal InsP levels in the brain by adenosine agonists. Because A_3 receptors appear to trigger PLC activation (12), we investigated the ability of IB-MECA, Cl-IB-MECA, and other chemically related and unrelated A_3 receptor agonists to stimulate hydrolysis of PIP_2 in the brain.

[1] Y. Shin, W. Daly, and K. A. Jacobson, unpublished observations.

Materials and Methods

Compounds. The following adenosine receptor agonists were used: IB-MECA, Cl-IB-MECA, DBXRM, NECA, APNEA, SPA, and DBXR. The chemical structures of these nucleosides are shown in Fig. 1. These derivatives were previously characterized for their A_3 receptor affinity and intrinsic activity by both radioreceptor-binding studies with A_1, A_{2A}, and A_3 radioligands and adenylyl cyclase inhibition in cell lines stably transfected with rat A_3 receptor cDNA (9, 17, 29).

IB-MECA, Cl-IB-MECA, DBXRM, SPA, and DBXR were synthesized as previously reported (16, 17, 29, 30). NECA, APNEA, and the xanthine derivatives CPX and XAC were obtained from Research Biochemicals International (Natick, MA); BWA1433 was a kind gift of Dr. Susan Daluge (Burroughs-Wellcome Co., Research Triangle Park, North Carolina).

Animals. Male Sprague-Dawley rats (2 months old) were used (Charles River, Calco, Italy). All animals were housed in a 12-hr light/dark cycle in rooms maintained at controlled temperature (22 ± 2°) and at standard humidity. Food and water were available ad libitum.

Assay of [^{3}H]-InsPs. Rat brain slices have been used extensively to characterize modulation of phosphoinositide transduction pathways by a variety of neurotransmitters (27, 28, 31, 32). Accumulation of [^{3}H]-labeled InsPs in rat cerebral slices was measured as described by Berridge *et al.* (33), with modifications according to Balduini *et al.* (34). Rats were killed by decapitation. Hippocampi and striata were rapidly dissected on ice and placed in carboxygenated (95% O_2/5% CO_2) KHB in the presence of ADA (Boehringer Mannheim, Germany) to remove endogenous adenosine. The KHB/ADA buffer contained NaCl (120 mM), KCl (4.7 mM), $CaCl_2$ (1.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), glucose (11.7 mM), $NaHCO_3$ (25 mM), and ADA (2 IU/ml), previously equilibrated to pH 7.4 with O_2/CO_2 (95%/5%).

Cerebral slices (350 × 350 μm) were cut using a McIlwain tissue-chopper and immediately suspended in fresh KHB/ADA. After two 15-min washes at 37° under slow shaking, slices were incubated for 1 hr at 37° with *myo*-[^{3}H]inositol (3 μCi/ml; 1 ml of gravity-sedimented cerebral slices in 10 ml of KHB/ADA) under sustained shaking to facilitate *myo*-[^{3}H]inositol incorporation. Before each experiment, *myo*-[^{3}H]inositol was purified by chromatography on 0.75 ml of a 50% (w/v) slurry of Ag-1 anion exchange resin (Bio-Rad Laboratories, Richmond, CA; 100–200 mesh) in the formate form. To remove the unincorporated *myo*-[^{3}H]inositol, slices were washed twice with KHB/ADA and once with KHB containing ADA and LiCl (7.5 mM) isotonically substituted for NaCl to inhibit the inositol-1-monophosphatase and thus permit detection of reaction products (KHLi/ADA). Fifty microliters of gravity-sedimented slices were then added to Beckman Bio-Vials containing the various adenosine receptor ligands in KHLi/ADA (final volume, 300 μl). Vials were gassed (95% O_2/5% CO_2), capped, and shaken at 37° for 1 hr. Previous experiments had shown that accumulation of [^{3}H]InsPs in rat brain slices was linear up to 90 min (34). In selected experiments, the effect of GDPβS on InsP formation was tested. Incubation was stopped by the addition of 940 μl of a chloroform/methanol mixture (1:2 w/v); 310 μl of chloroform and 310 μl of doubly distilled water were then added to cause separation of phases.

After centrifugation at 1000 × *g* for 10 min, 750 μl of the upper aqueous phase, representing the accumulated *myo*-[^{3}H]inositol content, was diluted to 3 ml with doubly distilled water and passed over 0.75 ml of a 50% (w/v) slurry of Ag-1X8 anion exchange resin. After seven to nine washes of the resin columns with 3 ml of 5 mM *myo*-inositol, InsPs were eluted with 2 ml of 1 M ammonium/formate/0.1 M formic acid. The eluate was then added to 8 ml of Luma Gel scintillation liquid (Packard Instruments, Groningen, The Netherlands) and counted in a Packard scintillation spectrometer with an

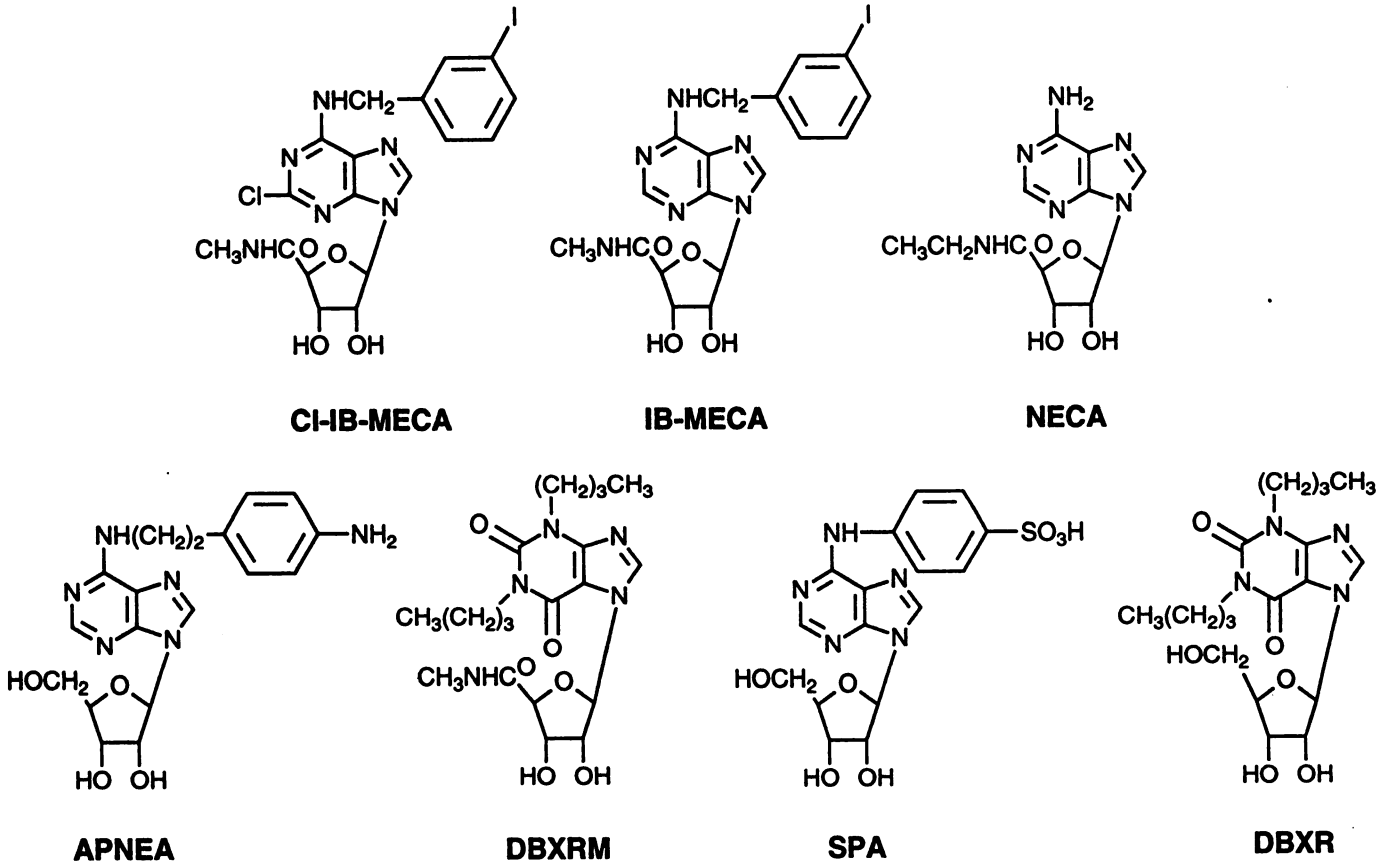

Fig. 1. Structures of adenosine agonists used in this study. See Materials and Methods for full chemical names.

efficiency of 60%. Two hundred microliters of the lower lipidic phase were added to 8 ml of the same scintillation liquid to determine the amount of *myo*-[^{3}H]inositol incorporated into membrane phospholipids (mostly as phosphatidylinositol; Ref. 35). Data were calculated as dpm/10^4 dpm incorporated (to correct for incorporation of *myo*-[^{3}H]-inositol into phospholipids) and expressed either as a percentage of basal (unstimulated) InsP formation or as a percentage of maximally stimulated InsP formation.

Assay of adenylyl cyclase activity. Adenylyl cyclase activity was assayed as previously described (36) in rat striatal preparations by the conversion of [^{32}P]ATP (NEN-DuPont, Italy) to [^{32}P]cAMP. Briefly, tissues were homogenized (10 strokes with a Teflon-glass potter) in 10 mM Tris·HCl, pH 7.4, containing 0.32 M sucrose and 2 mM EDTA. Homogenates were centrifuged (10 min at 800 × *g*) to pellet nuclei, and supernatants were collected and centrifuged again (25 min at 11,500 × *g*) to pellet membranes. Pellets were resuspended in 80 mM Tris-maleate (2 mM dithiothreitol, pH 7.4), and aliquots (~40 μg of protein) were incubated with Cl-IB-MECA in medium containing 0.5 mM ATP, 0.2 mM EGTA, 2 mM $MgCl_2$, 1 mM cAMP, 80 mM Tris-maleate, pH 7.4, 10 μM GTP, and 0.2 mM papaverine to inhibit cAMP phosphodiesterases; a regenerating system consisting of 6.3 mM creatine phosphate and 60 μg/sample creatine phosphokinase; 10^4 cpm/sample [^{3}H]cAMP (NEN-DuPont, Italy) to measure chromatographic recovery; 1 μCi/sample [^{32}P]ATP; and 2 IU/ml of ADA. To better evaluate possible inhibitory effects of Cl-IB-MECA on cAMP formation, adenylyl cyclase activity was increased by the addition of the direct activator forskolin (1 μM final concentration). After a 15-min incubation at 33°, samples were stopped by addition of a "stopping solution" containing 2% sodium dodecyl sulfate, 1.3 mM cAMP, and 40 mM ATP and boiled for 4 min to denature proteins. [^{32}P]cAMP was then purified by double-column chromatography on AG-50X4 (Bio-Rad Laboratories, Richmond, CA) and alumina columns as previously described (36). Results were calculated as pmol of cAMP/min/mg of protein and expressed as percent of forskolin-stimulated adenylyl cyclase activity.

Analysis of data. Concentration-response curves to agonists were analyzed by computer-assisted analysis by using Allfit (37). This analysis allows to verify if experimental data describe a sigmoidal concentration-effect relationship and, if so, to obtain EC_{50} and E_{max} values. Nonparametric one-way analysis of variance was determined using the Kruskall-Wallis test. Statistical significance of the single concentration data was determined with the Student's *t* test. Parametric two-way analysis of variance was carried out using either the Bonferroni or Fisher correction.

Results

Seven nucleoside analogues were studied (Fig. 1), of which IB-MECA, Cl-IB-MECA, and DBXRM were novel A_3-selective agonists. Fig. 2 shows the effects of these A_3-selective agonists and NECA on InsP formation in rat striatal slices. IB-MECA and Cl-IB-MECA stimulated the hydrolysis of PIP_2 at very low concentrations (~10^{-7} M), and their effects on InsP formation were both concentration dependent and statistically significant (Fig. 2A). The stimulation was saturable at high concentrations. EC_{50} and E_{max} values in InsP formation by these and other adenosine analogues are reported in Table 1. The effects on PIP_2 hydrolysis may be compared with affinity at A_1, A_{2A}, and A_3 receptors and with the ability to inhibit adenylyl cyclase activity via cloned rat A_3 receptors. The rank order of potency on InsP formation demonstrated by the selective agonists was consistent with their relative affinities in radioligand binding at rat A_3 receptor, as determined by displacement of specific binding of

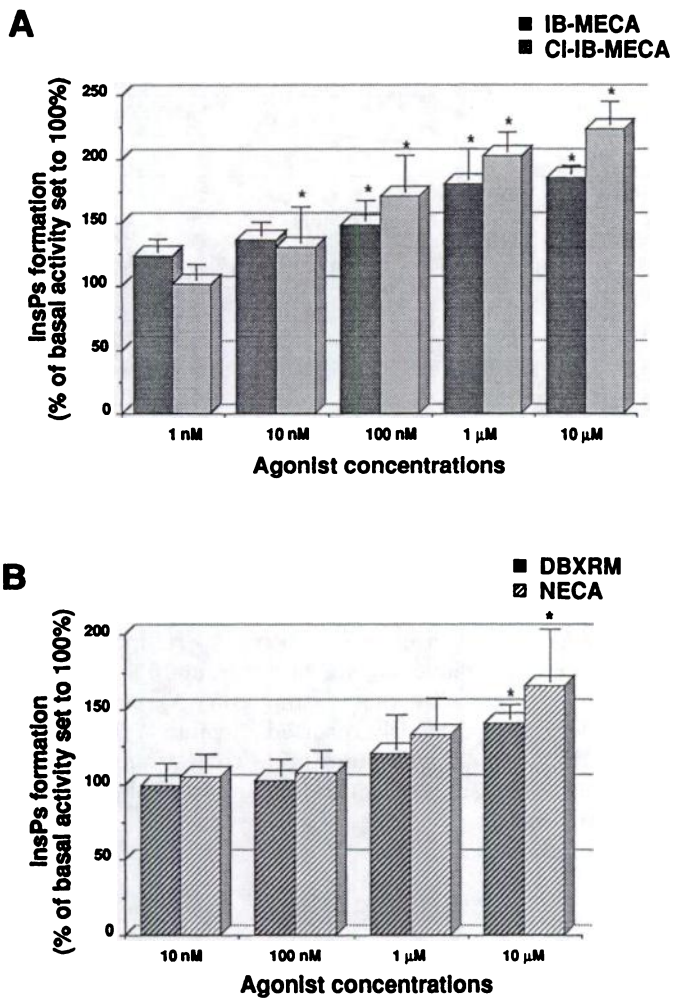

Fig. 2. Effects of (A) IB-MECA, Cl-IB-MECA, (B) DBXRM and NECA on PIP_2 hydrolysis in rat striatal slices. *myo*-[^{3}H]inositol-labeled slices were incubated with the various adenosine analogues at the concentrations reported on the *x*-axis. InsPs formation was measured as described in Materials and Methods. Results are expressed as percent of basal PIP_2 hydrolysis set to 100%. *, $p < 0.04$, with respect to basal InsP formation.

either [^{125}I]APNEA or [^{125}I]AB-MECA in CHO cells stably transfected with rat A_3 receptor cDNA (9, 17, 29). K_i values using these two radioligands for the same adenosine analogues as binding inhibitors at the cloned rat A_3 receptor were found to be nearly identical (16). The EC_{50} values in PLC activation were very similar to IC_{50} values in inhibiting adenylyl cyclase activity in CHO cells (17, 29).

The weaker A_3 agonists DBXRM and NECA also stimulated InsP accumulation in rat striatal slices (Fig. 2B). The EC_{50} values were higher than those for the N^6-benzyladenosine analogues (Table 1); both compounds appeared to stimulate PLC, with less than full efficacy in comparison with the N^6-benzyladenosine derivatives IB-MECA and Cl-IB-MECA.

Modest, although statistically significant, stimulations of PIP_2 hydrolysis were obtained with other adenosine receptor agonists (Table 1). Stimulation of InsP accumulation induced by 10 μM APNEA, SPA, or DBXR ranged between 23% and 48% over control, i.e., less than full efficacy. Also, the effects were concentration dependent (data not shown).

Based on all these results (Fig. 2 and Table 1), the potency order profile for adenosine analogues on PIP_2 hydrolysis in rat striatal slices was: Cl-IB-MECA ≥ IB-MECA ≫ DBXRM ≥ NECA ≥ APNEA > SPA = DBXR, which is consistent with the affinities at the adenosine A_3 receptor subtype (9, 17, 29).

To assess the involvement of G proteins in InsP formation elicited by adenosine receptor agonists, experiments were performed in the presence of the G protein inhibitor GDPβS. Although in control brain slices, IB-MECA potently stimulated InsP accumulation, the concomitant exposure of slices to either 100 μM or 1 mM GDPβS almost completely abolished IB-MECA effects on PIP_2 turnover (Fig. 3). These results demonstrate that in rat brain slices, stimulation of PLC activity by adenosine analogues occurs through a G protein.

Unlike the A_1, A_{2A}, and A_{2B} receptor subtypes, the rat A_3 receptor has been reported to be insensitive to xanthine antagonists (1, 5, 9). On this basis, we evaluated the ability of a number of xanthines to antagonize InsP accumulation induced by maximal IB-MECA concentrations in rat striatal slices (Table 2). CPX did not antagonize this effect at all. Very modest, although statistically significant, reductions of IB-MECA-stimulation of InsP formation were obtained with the potent $A_1/A_{2A}/A_{2B}$ antagonist XAC and with the acidic xanthine BWA1433 at high concentrations (11). The low antagonistic activity of XAC and BWA1433 on IB-MECA stimulation of PIP_2 hydrolysis is in agreement with previous studies of affinity at the rat A_3 receptor (9, 11). In experiments reported in Table 2, xanthine derivatives were tested at the 100 μM concentration; however, no significant antagonism of IB-MECA-elicited stimulation of PIP_2 hydrolysis was observed with any compound in a concentration range of 10 nM to 10 μM (data not shown).

Consistent with the present results, significant expression of A_3 receptors was demonstrated in mouse striatum using the high affinity radioligand [^{125}I]AB-MECA (18). Saturable binding to A_3 receptors in other brain regions, such as hippocampus and cortex, was also demonstrated. We have therefore evaluated the ability of IB-MECA to modulate InsP formation in rat hippocampal slices (Fig. 4) showing a significant stimulation of InsP formation in a concentration-dependent fashion (Fig. 4A; EC_{50}, 25.6 nM, %CV, 42.3). The maximal effect (143% over basal InsP formation) was lower with respect to that obtained in rat striatum, according to estimated lower levels of A_3 receptor in hippocampus (18). As in the striatum, hippocampal stimulation of InsP accumulation by IB-MECA was not antagonized by XAC tested over a wide range of concentrations at which A_1, A_{2A}, A_{2B} receptors should be blocked (Fig. 4B) because the K_i values of XAC at all three receptors are <100 nM (2). Only at 100 μM XAC was significant antagonism observed.

Curiously, in the presence of 1 μM XAC, a significant increase of IB-MECA stimulation of InsP formation was detected in hippocampal slices. A similar, although quantitatively lower, effect of xanthine antagonists was obtained in striatal slices. In the striatum, the A_1 antagonist CPX at a concentration of 0.1 μM potentiated PLC activation induced by 1 μM IB-MECA (178 ± 8% over basal InsP formation in the presence of both IB-MECA and CPX versus 157 ± 6% in the presence of IB-MECA alone). Such lower degree of potentiation with respect to hippocampal slices (Fig. 4B) is consistent with the lower density of A_1 receptors in the striatum.

The ability of A_3 receptors to modulate adenylyl cyclase

TABLE 1

Affinity at rat brain A_1, A_{2A}, and A_3 receptors; inhibition of adenylate cyclase; and effects on PIP_2 hydrolysis for the adenosine agonists used in the study

	Affinities in radioligand binding assays[a]			Cyclase Inhibition[a,e]	PIP_2 hydrolysis[f]	
	K_i A_1[b]	K_i A_{2A}[c]	K_i A_3[d]	IC_{50}	EC_{50}	E_{max}
		nM		nM	nM	%
Cl-IBMECA	820	470	0.33 ± 0.08	66.8	46.4 ± 9.6	240 ± 5
IB-MECA	54	56	1.1 ± 0.3	90.0	184 ± 65	192 ± 11
NECA	6.3	10.3	113 ± 34	5500	1241 ± 25	170 ± 34
APNEA	14	172	116 ± 18	N.D.	N.D.	148 ± 12[g]
DBXMR	37,300	$>10^{-4}$ M	229 ± 27	18,000	986 ± 138	140 ± 11
SPA	74	8900	526 ± 142	N.D.	N.D.	123 ± 9[g]
DBXR	2250	4250	1400 ± 160	44% maximum at 100,000	N.D.	129 ± 6[g]

[a] Values from Refs. 9, 17, 30.
[b] Displacement of specific [^{3}H]N^6-phenylisopropyladenosine binding from rat brain membranes.
[c] Displacement of specific [^{3}H]CGS 21680 binding from rat striatal membranes.
[d] Displacement of specific binding of [^{3}H]APNEA or [^{125}I]AB-MECA from membranes of CHO cells stably transfected with the rat A_3-cDNA (5).
[e] Inhibition of adenylyl cyclase activity in membranes of CHO cells stably transfected with the rat A_3-cDNA.
[f] In *myo*-[^{3}H]inositol-labeled rat striatal slices, as described in Materials and Methods. E_{max} values are expressed as percent of control InsPs formation set at 100%. N.D., not determined.
[g] Concentration = 10 μM. Although it was not possible to determine EC_{50} values, there was a statistically significant rise (six to nine experiments), with $p = 0.001$ for APNEA and DBXR and $p < 0.03$ for SPA (Student's *t* test).

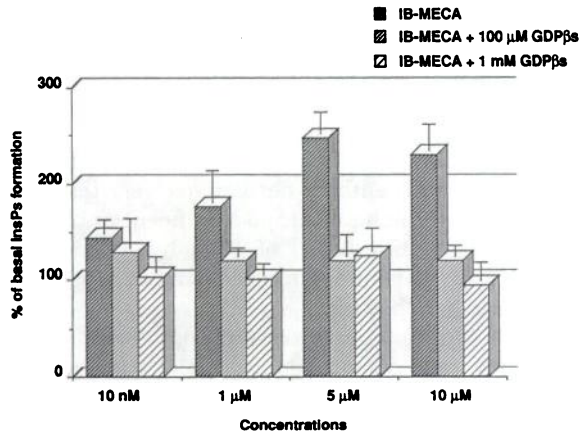

Fig. 3. Abolition of IB-MECA stimulation of InsPs formation by GDPβS in rat striatal slices. *myo*-[^{3}H]inositol-labeled slices were incubated with IB-MECA at the concentrations indicated on the *x*-axis in the absence or presence of either 100 μM or 1 mM GDPβS. PIP_2 hydrolysis was assayed as described in Materials and Methods (both curves, $p < 0.01$ by either Kruskall-Wallis test or Bonferroni).

TABLE 2

Effects of several xanthine derivatives on maximal IB-MECA stimulation of PIP_2 hydrolysis in rat striatal slices

Xanthine derivative	Percent of maximal IB-MECA-stimulated InsPs formation set to 100%
BWA1433	81.4 ± 6.5* (3)
XAC	84.3 ± 1.8**(3)
CPX	109.7 ± 7.5 (3)

Striatal slices were incubated with 10 μM IB-MECA in the absence (maximal InsPs formation) or presence of the indicated xanthine derivatives (all tested at 100 μM concentration). Levels of InsPs were determined as described in Materials and Methods. Results are expressed as percent of IB-MECA stimulated InsPs formation set to 100%. IB-MECA stimulation was 171.2 ± 12.8% with respect to basal InsPs formation. The number of determinations is given in parentheses.
* $P < 0.025$; ** $P = 0.001$, with respect to 10 μM IB-MECA, Student's *t* test.

activity was tested by measuring the concentration response to Cl-IB-MECA on forskolin-stimulated cAMP formation in striatal membranes (Fig. 5). The effect of Cl-IB-MECA on cyclase activity either alone or with CPX indicated a trend toward a subtle inhibition at concentrations of 10^{-10} to 10^{-6} M. This inhibitory effect therefore appears to be related to the activation of the xanthine-insensitive A_3 receptor. An increase of cAMP production was observed at 10^{-5} M (Fig. 5), perhaps reflecting activation of stimulatory A_2 receptors consistent with the previously reported K_i value of 0.47 μM for Cl-IB-MECA at A_{2A} receptors (17). This stimulation was abolished by CPX at a concentration known to block both A_1 and A_2 receptors.

Discussion

Activation of A_3 receptors was previously demonstrated to inhibit adenylyl cyclase activity in cell lines transfected with the rat brain A_3 receptor cDNA (5, 6, 9, 17, 29). However, until the present study the relevance of this and other transduction mechanisms to A_3 receptor function in the intact brain has been totally unknown. Forskolin-stimulated adenylyl cyclase activity in rat striatum was only very weakly inhibited by the A_3 receptor agonist Cl-IB-MECA, whereas PLC was stimulated in a dose-dependent fashion by A_3 receptor activation. A variety of selective A_3 receptor agonists were shown to stimulate PIP_2 hydrolysis and InsP generation in rat brain slices. Extensive evidence strongly suggests that these effects are mediated by the recently cloned G protein-coupled A_3 receptor. First, there is a good correlation between the rank order of potency on PLC stimulation for a variety of agonists and their previously demonstrated potency in both A_3 receptor binding and A_3 receptor-mediated adenylyl cyclase inhibition (9, 17, 29). Second, stimulation of PIP_2 hydrolysis by the selective A_3 agonist IB-MECA is completely abolished by the G protein inactivator GDPβS, which confirmed the involvement of a G protein-coupled receptor. Third, modulation of PIP_2 breakdown is only modestly affected by a number of xanthine derivatives, which is in agreement with the previously demonstrated xanthine insensitivity of the cloned rat A_3 receptor (5, 9, 11) and with the inability of CPX to reverse the *in vivo* effects of IB-MECA (18). Fourth, PLC stimulation is higher in rat striatum versus the hippocampus, which is consistent with the estimated levels of A_3 receptors in these two brain areas (18). Thus, our results show for the first time that stimulation of PLC activ-

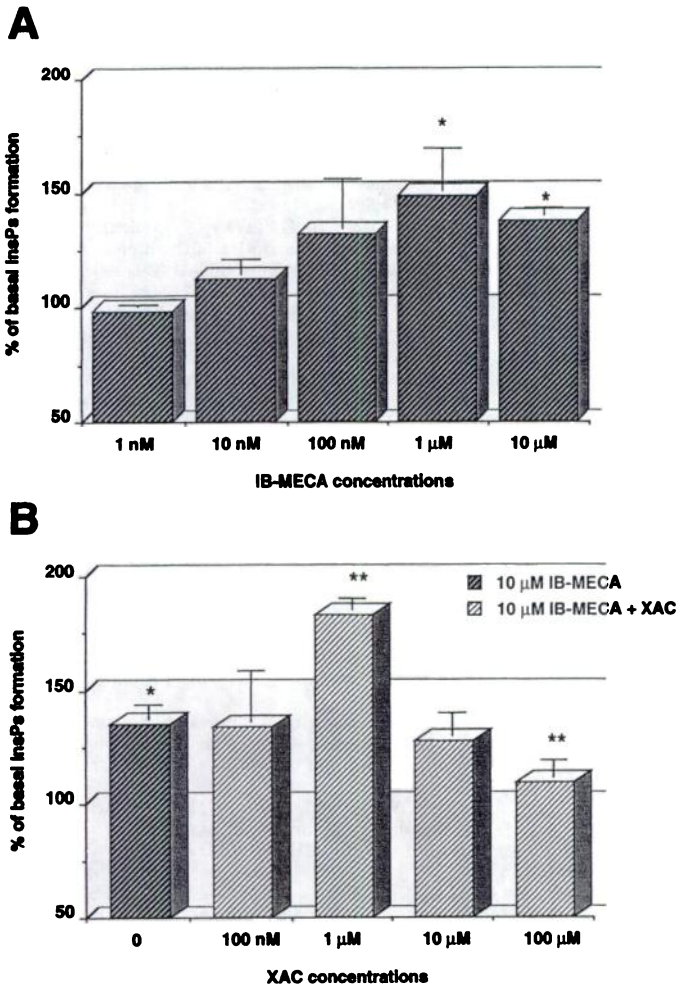

Fig. 4. IB-MECA stimulated InsPs formation in rat hippocampal slices and effect of XAC. A, *myo*-[^{3}H]inositol-labeled slices were incubated with IB-MECA at the concentrations indicated on the *x*-axis. PIP_2 hydrolysis was assayed as described in Materials and Methods. *, $p <$ 0.02 with respect to basal InsPs formation. B, *myo*-[^{3}H]inositol-labeled slices were incubated with 10 μM IB-MECA in the absence or presence of graded XAC concentrations. InsP formation was assayed as described in Materials and Methods. *, $p < 0.01$ with respect to basal InsP formation; **, $p < 0.02$ with respect to 10 μM IB-MECA.

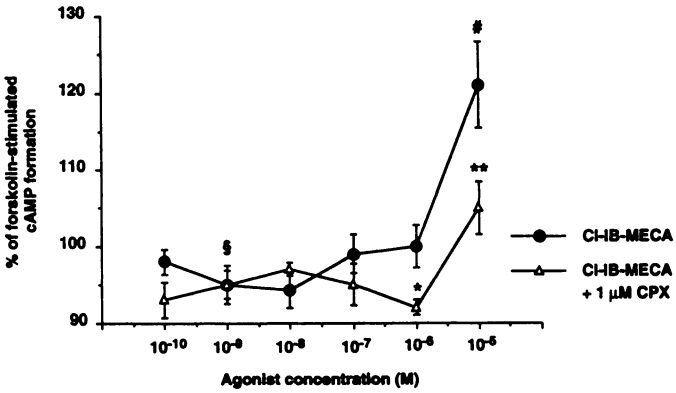

Fig. 5. Effect of Cl-IB-MECA on forskolin-stimulated cAMP formation in rat striatum. Forskolin-stimulated adenylyl cyclase activity was assayed in the presence of the Cl-IB-MECA concentrations indicated on the *x*-axis in the absence or presence of 1 μM CPX. Results are expressed as percent of control forskolin-stimulated activity. Each value is the mean ± standard error of eight experiments run in triplicate. Cl-IB-MECA alone: #, $p < 0.003$ with respect to corresponding control, Bonferroni test; Cl-IB-MECA + CPX: § and *, $p < 0.03$ with respect to corresponding control; **, not statistically different from control, Fisher's test; and $p < 0.004$ with respect to #, posthoc test of a two-way analysis of variance.

ity represents a principal transduction mechanism for A_3 receptors in mammalian brain.

A discrepancy for A_3 agonists between the IC_{50} values in the adenylyl cyclase functional assay and the K_i values in binding assay (i.e., the latter being lower by a factor of ≥40) was previously noted (Table 1; Refs. 17 and 29). A similar discrepancy applies to stimulation of InsP formation by N^6-benzyladenosine-5′-uronamides (Table 1). Such consistent differences between binding and functional dose-response curves are common to a number of other receptors and are due to differences in the experimental systems used for evaluating binding and second messenger formation and complex protein interactions that moderate the functional effects.

In the present study, the xanthine riboside derivative DBXRM and the adenosine analogue NECA stimulated InsP accumulation in rat striatal slices (Fig. 2B), with the EC_{50} values in the micromolar range (Table 1). These results are in agreement with the relative binding affinities of these compounds at cloned rat A_3 receptors (9, 29). Although lacking an adenine moiety, the xanthine riboside DBXRM was shown to be a full agonist of intermediate potency in A_3-mediated inhibition of adenylyl cyclase (29). However, in PLC activation (Fig. 2A; Table 1), both DBXRM and NECA behaved as partial agonists in comparison with the efficacies of IB-MECA and Cl-IB-MECA (Fig. 2B; Table 1). This apparent discrepancy might be related to differences in the experimental model (i.e., the transfected cloned A_3 receptor for the cyclase assay and, in the present study, rat brain slices). Specifically for the adenosine A_3 receptor, this phenomenon has already been reported (7); the efficacy of various agonists at human A_3 receptors differs by as much as 2-fold. This includes at least one partial agonist that was previously classified as a full agonist at other adenosine receptors.

The lower potency of APNEA, SPA, or DBXR with respect to the A_3-selective IB-MECA and Cl-IB-MECA is consistent with their lower affinity toward the cloned rat A_3 receptor (Table 1; Ref. 9). Interestingly, the nonselective and relatively weak agonist DBXR, which was previously reported to behave as a partial agonist in the A_3-mediated inhibition of adenylyl cyclase (9), in this study also did not behave as a full agonist with respect to its effects on InsP formation in brain slices.

Only very high concentrations (10–100 μM) of certain xanthines (XAC and BWA1433) were effective in antagonizing the PLC activation. The K_i value of XAC in radioligand binding at cloned rat brain A_3 receptors is 29 μM (11). BWA1433 is a xanthine derivative containing an acidic phenyl substitution in the C8 position, which has been reported to bind with intermediate affinity at cloned sheep and human A_3 receptors (10) and very weakly at rat A_3 receptors (11). Thus, these findings support the involvement of A_3 receptors in the PLC effects.

Adenosine A_1 receptors were previously demonstrated to inhibit histamine-activated PLC activity in rat brain (28). It could be hypothesized that the "paradoxical" effect, in which XAC or CPX stimulated PLC, could be related to antagonism of A_1 receptor-mediated inhibition. Such antagonism would

lead to a net increase of stimulation of PLC activity in the presence of 10 μM IB-MECA. The disappearance of the stimulation at higher XAC concentrations might be due to antagonism of the A_3 receptor-mediated effect, which is in agreement with the K_i values of this antagonist at cloned rat A_3 receptors (11). To further support this hypothesis, the highly selective A_1 receptor antagonist CPX also potentiated stimulation of PIP_2 hydrolysis by IB-MECA in slices (data not shown).

We speculate that A_3 receptor-mediated increases of inositol phosphates in the ischemic brain may contribute to neurodegeneration by raising intracellular calcium levels. In general, PLC activation leads to a rise in intracellular calcium. "Calcium-mobilizing" receptors in the central nervous system have been involved in pathological changes associated with excessive intracellular calcium concentrations, e.g., aging- and ischemia-associated neuronal cell death (24–26, 38). It has been known for a long time that adenosine levels in the brain are massively increased during ischemia and hypoxia (39, 40). Because acute administration of the selective A_3 agonist IB-MECA *in vivo* aggravated ischemia-associated neuronal degeneration and enhanced mortality in gerbils (20), it is likely that, in the ischemic brain, activation of cerebral A_3 receptors by endogenous adenosine may contribute to neurodegeneration through increases of intracellular InsP levels and consequent calcium mobilization. Thus, an A_3 antagonist might prove to be cerebroprotective on acute administration in stroke.

A cerebroprotective effect against ischemia-associated mortality and neuronal destruction was demonstrated in gerbils after chronic treatment with IB-MECA (20). Such regimen-dependent inversion of IB-MECA-induced effects might be related to adaptive changes of brain A_3 receptors after chronic exposure to agonists (agonist-induced desensitization), in a similar way to that hypothesized for other adenosine receptor subtypes (41–46). Future studies aimed at evaluating A_3 receptor-mediated PLC stimulation in the brains of animals chronically treated with A_3 agonists will clarify this important point.

In conclusion, the present results provide a molecular mechanism that might be the basis of the previously described central nervous system effects of adenosine A_3 receptor agonists. Moreover, the present data support the hypothesis that adenosine A_3 receptors may play a role in the pathophysiology of cerebral ischemia (and, possibly, of other neurological disorders) and that they may represent a novel target for drug development.

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