α,β-Methylene ATP enhances P2Y4 contraction of rabbit basilar artery

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Submitted 29 September 2003; accepted in final form 5 December 2003

Miyagi, Yasushi, and John H. Zhang. α,β-Methylene ATP enhances P2Y4 contraction of rabbit basilar artery. Am J Physiol Heart Circ Physiol 286: H1546–H1551, 2004; 10.1152/ajpheart.00926.2003.—Interactions between different selective P2 receptor agonists have been used as tools to identify different P2 receptor subtypes. In the present study, we examined the P2 receptor subtypes and the mechanisms of potentiation of UTP contraction (P2Y contraction) by α,β-methylene ATP [2-carboxyoxypiperazin-4-yl)propyl-1-phosphanoic acid (CPP), a P2X agonist] using isometric tension in the denuded rabbit basilar artery. We made the following observations: 1) a predominant P2X receptor contraction was observed in the rabbit ear artery by the rank order of CPP > ATP > UTP; 2) functional P2Y receptors were observed in the rabbit basilar artery by the rank order of UTP > ATP = CPP; 3) CPP potentiated UTP-, ATP-, and ATPγS-induced contractions, possibly by activation of P2Y4 receptors because ATPγS does not activate P2Y6 receptors; and 4) ectonucleotidase did not play a predominant role in the potentiative effect of CPP because Evans blue, Ca2+-free medium, or divalent cation Ni2+ did not affect the effect of CPP. Evans blue potentiated the contraction by UTP but not by ATP or ATPγS. We conclude that CPP enhanced P2Y4-mediated contraction in the rabbit basilar artery, and the influence by ectonucleotidases on CPP-potentiation remains unclear.

P2 receptors in the vascular smooth muscle and endothelium contribute immensely to the regulation of cerebral circulation (15, 24, 47), especially under pathological conditions such as traumatic brain injury (12), cerebral ischemia (20, 26), and cerebral vasospasm after subarachnoid hemorrhage (41, 48–50). Two P2 receptor families have been established, including subclasses of P2X and P2Y receptors (4, 5). Among them, P2Y receptors are actively involved in the regulation of cerebral arterial tone, and P2Y1, P2Y2, P2Y4, and P2Y6 have all been identified in cerebral arteries (6, 15, 16, 21, 24, 26). Although many subtypes of P2Y receptors are identified in detail at the molecular level (21, 44), the physiological function of these P2Y receptors in cerebral arteries has yet to be explored.

To study the pharmacology of P2 subtypes, the cross-desensitization method has been employed using selective P2 receptor agonists (33, 39, 42). In our preliminary experiments, however, we found that some agonists such as α,β-methylene ATP [2-carboxyoxypiperazin-4-yl)propyl-1-phosphanoic acid (CPP)], one of the selective agonists for P2X receptor, enhanced, rather than desensitized, the contractile response to UTP, an agonist of P2Y receptors. Similar observations have been reported in literature on peripheral vascular systems (22, 27, 34, 43). We used a combination of agonist potency and agonist sensitization to identify the P2 receptor subtypes and an influence by ectonucleotidases on this potentiation in the rabbit basilar artery.

METHODS

Isometric tension study. The Animal Care and Use Committee at the University of Mississippi Medical Center approved all the procedures for using rabbit tissues in the present study. Thirty male New Zealand White rabbits were anesthetized with an injection of thiopental (20 mg/kg iv) and euthanized by exsanguination. The basilar arteries (and ear arteries in some rabbits) were removed and cut into 3-mm rings in a dissecting chamber filled with modified Krebs-Henseleit bicarbonate solution containing (in mM) 120 NaCl, 4.5 KCl, 1 MgSO4, 27 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, and 10 dextrose and bubbled with 95% O2-5% CO2. We removed endothelial cells by gently rubbing the rings with a steel hook (9, 10, 28). The removal of endothelium was confirmed by a loss of relaxation to 30–50 μM acetylcholine (rings precontracted with 30 μM serotonin).

Rings were suspended at a resting tension of 400 mg (Ratno transducer, Radnoit Glass) between stainless steel hooks in a 10-ml water-jacketed tissue bath (Radnoit Glass) in modified Krebs-Henseleit bicarbonate buffer with 95% O2-5% CO2 at 37°C. Rings were incubated for 90 min until a stable rest tension was achieved, and the solution was changed every 20 min to remove metabolites. The tissues were challenged with 118 mM KCl twice at 30-min intervals before the experiments. The tension developed by serotonin application was shown as the ratio to 118 mM KCl. For studies in which CPP needed to be removed, the buffer solution was prewarmed and preaerated in a 37°C water bath, mixed with the agonists (UTP) at the designated concentration, and injected slowly into the water bath. Isometric force transducers were connected to arterial rings, and contraction was recorded with an eight-channel MacLab 8E and stored on a Power Macintosh computer.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA. Differences were considered to be significant when the P value was <0.05.

RESULTS

Potency order of nucleotides in the rabbit ear and basilar arteries. We compared the P2 receptors in peripheral (ear) artery and cerebral (basilar) arteries. The concentrations of each agonist were added from 10−8 to 10−3 M in an accumulated manner. Each dose was applied to the chamber for at least 5 min (or when contraction reached the plateau phase) before the second dose was administered. In the rabbit ear artery, the potency order of contraction induced by nucleotides was ATP > 2-methylthioATP > ATPγS, indicating a predominant role of P2X receptors (Fig. 1A) (19).

In the rabbit basilar artery, the rank order of contraction was UTP > ATP = CPP, indicating contractile function of P2Y receptors (Fig. 1B) (44). UTP was the most potent contractile agonist in the rabbit basilar artery, and the

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blue inhibit the activity of ectonucleotidase enzymes (35). We tested the effect of CPP in the presence of Evans blue and Ni\(^{2+}\) as well as in the absence of external Ca\(^{2+}\).

Evans blue, a potent inhibitor of ectonucleotidase, enhanced the contraction induced by UTP. Figure 2A summarizes the effect of CPP and Evans blue on UTP contraction. In these concentration-dependent contractile studies, CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of UTP was used. The accumulated concentration of UTP that was used was from 10\(^{-7}\) to 10\(^{-3}\) M. Both CPP and Evans blue enhanced UTP contraction \((P < 0.05\) vs. UTP alone, ANOVA), even though the effect of CPP was greater \((P < 0.05\) vs. UTP/Evans blue).

The potentiation effect of CPP and Evans blue on ATP contraction was tested in the rabbit basilar artery. In these concentration-dependent contractile studies (Fig. 2B), CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of ATP was used. The accumulated concentration of ATP was used was from 10\(^{-7}\) to 10\(^{-3}\) M. Both CPP and Evans blue potentiated ATP-induced contraction \((P < 0.05\) vs. ATP alone, ATP/Evans blue, respectively; Fig. 2B).

Another P2Y agonist, ATP\(_{\gamma}\)S, which is resistant to hydrolysis by ectonucleotidase (35), produced similar contractile action compared with UTP in the rabbit basilar artery (Fig. 2C). CPP potentiated the contraction induced by ATP\(_{\gamma}\)S either applied after (not shown) or before ATP\(_{\gamma}\)S administration (not shown). ATP\(_{\gamma}\)S did not produce additional contraction on top of ATP contraction in the presence of CPP (not shown), indicating that UTP and ATP\(_{\gamma}\)S share the same P2 receptor. Figure 2C summarizes the effect of CPP and Evans blue on ATP\(_{\gamma}\)S contraction. CPP but not Evans blue potentiated ATP\(_{\gamma}\)S contraction \((P < 0.05\) vs. ATP\(_{\gamma}\)S alone, ATP\(_{\gamma}\)S/Evans blue, respectively; Fig. 2C). In these concentration-dependent contractile studies (Fig. 2C), CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of ATP\(_{\gamma}\)S was used. The accumulated concentration of ATP\(_{\gamma}\)S that was used was from 10\(^{-7}\) to 10\(^{-4}\) M.

Figure 3A demonstrates that Evans blue enhanced UTP contraction but did not inhibit CPP-induced potentiation of UTP contraction (Fig. 3A). In combination with Ni\(^{2+}\) (1 \(\mu\)M), a Ca\(^{2+}\) entry blocker and an inhibitor of ectonucleotidase, Evans blue again failed to reduce CPP-induced potentiation of UTP contraction (Fig. 3B). Ni\(^{2+}\) (1 \(\mu\)M) reduced the plateau phase of UTP contraction, which is dependent on Ca\(^{2+}\) entry (Fig. 3B). There seems to be an interaction between Evans blue and Ni\(^{2+}\) because Evans blue restored at least partially the plateau force of UTP contraction (Fig. 3B). Finally, CPP potentiated UTP contraction in the presence (Fig. 4A) or absence of external Ca\(^{2+}\) (Fig. 4B). Because of reduced UTP contraction in the absence of external Ca\(^{2+}\), a high concentration of UTP (200 \(\mu\)M) was used.

**DISCUSSION**

We demonstrated the potency order of ATP \(\geq\) ATP\(_{\gamma}\)S \(\gg\) ATP = CPP = 2MeSATP in the contraction of the rabbit basilar artery. CPP, even though it did not produce any contraction by itself, enhanced the contractile effect of UTP, ATP, and ATP\(_{\gamma}\)S either when CPP was applied before or after the administration of UTP, ATP, and ATP\(_{\gamma}\)S. The mechanism of

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**Fig. 1.** Concentration-dependent contractions were obtained in endothelium-denuded rings of the rabbit ear \((A)\) and basilar arteries \((B)\). The contraction was normalized by a maximum contraction by KCl (118 mM), which was designated as 100%. The rank order of potency in the rabbit ear artery was (2-carboxypiperazin-4-yl)propyl-1-phosphanoic acid (CPP) \(\gg\) 2-methylthio-ATP (2MeSATP) \(>\) ATP \(>\) UTP. UTP failed to produce noticeable contraction (1 mM). In the rabbit basilar artery, the rank order of potency was UTP \(>\) ATP \(\geq\) 2MeSATP = CPP. Among them, ATP, 2MeSATP, and CPP failed to produce marked contraction at 100 \(\mu\)M.

**Response of the receptors to UTP might involve P2Y2, P2Y4, or P2Y6 receptors** (3, 35).

Maximum contraction and EC\(_{50}\) were not calculated because most agonists failed to reach maximum contraction at the highest dosages used except for CPP in the rabbit ear artery.

**Effect of CPP on UTP-induced contraction.** CPP (at 100 \(\mu\)M), which did not induce contraction in the rabbit basilar artery (Fig. 1B), potentiated the contraction induced by UTP either applied before (not shown) or after UTP administration (not shown) (also comparing UTP-induced contraction in Fig. 1B). The potentiation effect of CPP was rapid and reversible; indicating the acting site of CPP is at the receptor level but not at the intracellular level. CPP did not change the kinetics of UTP-induced contraction in the rabbit basilar artery, which remained rapid and easily reversible by washing out with buffer solution (not shown).

**Ectonucleotidase and CPP-induced potentiation.** Ca\(^{2+}\) depletion, divalent cation (Ni\(^{2+}\) > Mn\(^{2+}\) > Ca\(^{2+}\)), and Evans blue inhibit the activity of ectonucleotidase enzymes (35). We tested the effect of CPP in the presence of Evans blue and Ni\(^{2+}\) as well as in the absence of external Ca\(^{2+}\).

Evans blue, a potent inhibitor of ectonucleotidase, enhanced the contraction induced by UTP. Figure 2A summarizes the effect of CPP and Evans blue on UTP contraction. In these concentration-dependent contractile studies, CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of UTP was used. The accumulated concentration of UTP that was used was from 10\(^{-7}\) to 10\(^{-3}\) M. Both CPP and Evans blue enhanced UTP contraction \((P < 0.05\) vs. UTP alone, ANOVA), even though the effect of CPP was greater \((P < 0.05\) vs. UTP/Evans blue).

The potentiation effect of CPP and Evans blue on ATP contraction was tested in the rabbit basilar artery. In these concentration-dependent contractile studies (Fig. 2B), CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of ATP was used. The accumulated concentration of ATP that was used was from 10\(^{-7}\) to 10\(^{-3}\) M. Both CPP and Evans blue potentiated ATP-induced contraction \((P < 0.05\) vs. ATP alone, ATP/Evans blue, respectively; Fig. 2B).

Another P2Y agonist, ATP\(_{\gamma}\)S, which is resistant to hydrolysis by ectonucleotidase (35), produced similar contractile action compared with UTP in the rabbit basilar artery (Fig. 2C). CPP potentiated the contraction induced by ATP\(_{\gamma}\)S either applied after (not shown) or before ATP\(_{\gamma}\)S administration (not shown). ATP\(_{\gamma}\)S did not produce additional contraction on top of UTP contraction in the presence of CPP (not shown), indicating that UTP and ATP\(_{\gamma}\)S share the same P2 receptor. Figure 2C summarizes the effect of CPP and Evans blue on ATP\(_{\gamma}\)S contraction. CPP but not Evans blue potentiated ATP\(_{\gamma}\)S contraction \((P < 0.05\) vs. ATP\(_{\gamma}\)S alone, ATP\(_{\gamma}\)S/Evans blue, respectively; Fig. 2C). In these concentration-dependent contractile studies (Fig. 2C), CPP and Evans blue (both at 100 \(\mu\)M) were applied and incubated with rings for 20 min before the first dose of ATP\(_{\gamma}\)S was used. The accumulated concentration of ATP\(_{\gamma}\)S that was used was from 10\(^{-7}\) to 10\(^{-4}\) M.

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**DISCUSSION**

We demonstrated the potency order of UTP \(\geq\) ATP\(_{\gamma}\)S \(\gg\) ATP = CPP = 2MeSATP in the contraction of the rabbit basilar artery. CPP, even though it did not produce any contraction by itself, enhanced the contractile effect of UTP, ATP, and ATP\(_{\gamma}\)S either when CPP was applied before or after the administration of UTP, ATP, and ATP\(_{\gamma}\)S. The mechanism of
CPP-produced potentiation of UTP contraction was studied further, and we found that it was not predominantly by the inhibition of ectonucleotidase because the effect of CPP was not inhibited by divalent cation Ni\(^{2+}\)/Ca\(^{2+}\)-free medium, or Evans blue. In addition, CPP potentiated the contraction of Fig. 2. Summary of the effect of CPP and Evans blue on UTP (A), ATP (B), and ATP\(_7\)S (C) contractions. Evans blue (EB/UTP) potentiates the contraction by UTP (1 µM to 1 mM, *P < 0.05, ANOVA). CPP (CPP/UTP) markedly enhanced the contraction by UTP (1 µM to 1 mM, *P < 0.05, ANOVA). UTP produced more contraction in the presence of CPP than in the presence of Evans blue (*P < 0.05, ANOVA). ATP\(_7\)S produced stronger contraction than ATP. CPP enhanced contractions to ATP and ATP\(_7\)S. Evans blue did not affect either ATP\(_7\)S contraction or ATP contraction.

Fig. 3. Real tracings showed examples of KCl (118 mM contraction, which was followed by UTP contraction). A: UTP contraction was slightly enhanced by Evans blue. In the presence of UTP and Evans blue, CPP potentiated UTP contraction markedly. B: example showing UTP contraction in the presence of Ni\(^{2+}\) (1 mM), which blocks Ca\(^{2+}\) entry. Ni\(^{2+}\) reduced the plateau phase UTP contraction. Evans blue did not cause noticeable contraction in the presence of Ni\(^{2+}\). CPP in the presence of Ni\(^{2+}\) and Evans blue enhanced UTP contraction. Values are means ± SE.

CPP-produced potentiation of UTP contraction was studied further, and we found that it was not predominantly by the inhibition of ectonucleotidase because the effect of CPP was not inhibited by divergent cation Ni\(^{2+}\), Ca\(^{2+}\)-free medium, or Evans blue. In addition, CPP potentiated the contraction of...
ATP\(_2\)S, which is resistant to ectonucleotidase. These results indicate that CPP potentiates P2Y contraction in the rabbit basilar artery.

Six cloned human P2Y receptors have been identified, including P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, and P2Y12. These P2Y receptors are widely distributed in most human tissues (39, 44). In the cerebral circulation, P2Y1, P2Y2, P2Y4, and P2Y6 are all identified and believed to contribute to the regulation of cerebral blood flow and vascular tone (6, 15, 16, 21, 24, 26). It is unlikely that the functional P2Y1 receptor is involved in the rabbit basilar artery because the agonist potency for P2Y1 is 2MeSATP > ADP > ATP (39), which is opposite of the agonist potency observed in the present study. Because UTP contraction involves P2Y2, P2Y4, and P2Y6, we used a combination of agonist potency with receptor sensitization to distinguish the receptor subtypes. As summarized above, the potency order is UTP ≥ ATP\(_2\)S >> ATP = CPP = 2MeSATP in the present study. It has been established that ATP is an equipotent agonist because UTP in contraction is mediated by the P2Y2 receptor subtype (rank order of P2Y2 is UTP = ATP > ATP\(_2\)S) (31, 32, 44). On the contrary, for the P2Y6 receptor, UDP is the most potent agonist, whereas UTP and ATP are weak agonists or inactive (31, 44). In the present study, UTP produced strong contractile effects, whereas ATP produced weak contractile effects in the rabbit basilar artery (Fig. 1B), suggesting that UTP-induced contraction was most likely mediated by P2Y4 (14), most unlikely mediated by P2Y6, and not mediated by P2Y2 (44). In addition, it has been established that ATP\(_2\)S activates P2Y2 and P2Y4 but not P2Y6 receptors (3, 35, 44). In the present study, ATP\(_2\)S was almost as equally potent as UTP and was much more potent than ATP, indicating that the receptor subtype is not P2Y2 and certainly not P2Y6. Furthermore, ATP\(_2\)S did not produce additional contraction on top of UTP contraction in the presence of CPP, indicating that UTP and ATP\(_2\)S share the same P2 receptor. All these results suggest that the contractions induced by UTP, ATP, and ATP\(_2\)S, and the potentiation effect of CPP on those contractions in the rabbit basilar artery, are mediated by P2Y4 receptors.

The mechanisms of CPP-induced potentiation of UTP contraction may be mediated by CPP inhibition of ectonucleotidase activity that breaks down extracellular nucleotides (35). Ectonucleotidases are plasma membrane-bound enzymes that dephosphorylate extracellular nucleotides, including UTP. ATP\(_2\)S and CPP are resistant to these enzymes (3, 39). Ca\(^{2+}\) depletion, divalent cations (Ni\(^{2+}\) > Mn\(^{2+}\) > Co\(^{2+}\)), and Evans blue inhibit the activity of ectonucleotidase enzymes (35). In the present study, however, Evans blue, Ca\(^{2+}\) depletion, and the divalent cation Ni\(^{2+}\) all failed to inhibit CPP-induced potentiation of UTP contraction. Furthermore, CPP enhanced the contraction by ATP\(_2\)S, which is resistant to ectonucleotidase. Even though Evans blue enhanced UTP contraction, which could be mediated by inhibition of ectonucleotidase, Evans blue did not inhibit CPP-induced potentiation, indicating that CPP-induced potentiation was not predominantly by inhibition of ectonucleotidase. However, Evans blue did not potentiate ATP- or ATP\(_2\)S-induced contractions, indicating either a lack of activity of ectonucleotidase in the rabbit basilar artery preparation or an inefficiency of Evans blue on ectonucleotidase inhibition. In one other study (1), UTP was degraded by rat vas deferens tissue, and the degradation was inhibited by Evans blue (100 μM). Evans blue (100 μM) greatly enhanced contractions elicited by the uracil nucleotides. The effect of Evans blue was not studied directly with ATP but in combination with α,β-methylene ATP, which reduced the contraction by ATP (1). In another study (2), 8,8'-[carbonylbis(imino-3,1-phenylenecarbonyl-imino)]-bis-(1,3,5)-naphthalenetrisulphonate (NFO23), suramin, pyridoxalphosphate-6-azophenyl-2',5'-disulphonate (iso-PPADS), trypan blue, and reactive blue 19, in contrast, caused only partial blockade, by 34–43% maximally; reactive blue 2 and reactive red 2 had no effect; and 6,6'-((1',4'-diylbisazo)-bis-4-amino-5-hydroxy-naphthalene-1,3-disulphonate (NH01) and Evans blue even enhanced the response to ATP. Overall, these results indicate that the action of CPP was not predominantly by inhibition of ectonucleotidase activity in the rabbit basilar artery and could be a combination of inhibition of ectonucleotidase and other mechanisms.

In the present study, we have shown a predominant functional P2Y4 contraction, which is responsive to UTP, ATP, and ATP\(_2\)S in the rabbit basilar artery. Interestingly, CPP produced strong contraction in the rabbit ear artery but did not induce contraction at all in the rabbit basilar artery, which is consistent with a previous study (36), indicating a dominant role of P2X receptors in peripheral but not cerebral arteries. In addition, CPP potentiated but did not desensitize P2Y4 contraction, consistent with several other reports in peripheral vascular systems (22, 27, 34, 43). This observation was in contrast with many other observations that showed that P2Y receptor agonists desensitize the contractile action of each other (35, 39). In cerebral vascular systems, extracellular nucleotides can be released from aggregated platelets (11) or lysed cells from blood clots (48). Therefore, multiple nucleotide receptors are supposed to be stimulated simultaneously by these nucleotides. To protect P2 receptors from being overly stimulated, several protective mechanisms were discovered, such as ectonucleotidase, which causes hydrolysis of nucleotides (19) as well as receptor internalization (35) and receptor desensitization (7, 42). Our observations indicate that protective mechanisms other than ectonucleotidase exist in the rabbit basilar artery. CPP might inhibit such protective mechanisms and facilitate the full activation of P2Y4 by UTP, ATP, and ATP\(_2\)S. Because CPP is also a contractile agent, its contraction could be potentiated by other agents. In the present study using the rabbit basilar artery, CPP did not produce any contraction (Fig. 1), which excluded P2X receptors in the basilar artery. Second, CPP not only potentiated UTP contraction but enhanced contractions to ATP and ATP\(_2\)S as well. It seems unlikely that all of these agents (ATP, UTP, and ATP\(_2\)S) potentiate CPP contraction and use the same unknown mechanism because it would be more unlikely that these three agents enhance CPP contraction using different mechanisms. Finally, if CPP causes contraction by activating P2X receptors, it should not enhance UTP contraction in the absence of external Ca\(^{2+}\). We believe that it is likely that CPP enhanced contractions by UTP, ATP, and ATP\(_2\)S.
constriction (24). However, the contractile response in human omental arteries was mediated by P2X and P2Y but not by P2Y6 receptors (24), and P2Y6 does not exist in human coronary arteries (23). In rat cerebral arterioles, functional P2X1 and P2Y2 regulate vascular tone (15), but in the rat basilar artery, P2Y6 predominately contributes to contraction (25). A striking example of species difference is that ATP is an agonist at the rat P2Y4 receptor but is an antagonist at the human P2Y4 receptor (18). Most investigators used rat cerebral arteries to study the subtypes and functions of P2 receptors (6, 8, 15, 20, 21, 25, 26, 29, 40, 45–48). Some used cerebral arteries from cows (30, 50) or dogs (40), and some used human cerebral arteries (23, 24). Except for one early article (13) that used the rabbit pial artery, other studies have used rabbit peripheral arteries, including P2Y2 in the rabbit coronary (38) and carotid arteries (37). A study (24) using human cerebral arteries reported that the selective P2Y6 receptor agonist UDP by itself was the most potent of all the agonists. The agonist potency, UDP*S > α,β-methylene ATP > UTP*S > ATP*S > ADP*S > ATP = 0, indicated the presence of contractile P2X1, P2Y2, P2Y4, and P2Y6, but not P2Y1, receptors. RT-PCR analysis of endothelium-denuded human cerebral arteries demonstrated P2X1, P2Y1, P2Y2, and P2Y6 receptor mRNA expression. P2Y4 receptor mRNA was barely detectable in the cerebral arteries (24). The results from the above-mentioned study (24) using human cerebral arteries are different from those of ours in that we did not obtain any function of P2X1 and P2Y4 that plays a dominant role in the rabbit basilar artery. Thus to establish the P2Y receptor as a therapeutic target, human cerebral arteries, collected from different locations (basilar vs. middle cerebral) and at different sizes (major vs. pial), should be examined.

GRANTS

This study was partially supported by an American Heart Association Buehler Foundation for Stroke Award and by National Institutes of Health Grants NS-45694 and HD-43120 (to J. H. Zhang).

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