Potentiation of P2X1 ATP-Gated Currents by 5-Hydroxytryptamine 2A Receptors Involves Diacylglycerol-Dependent Kinases and Intracellular Calcium

Ariel R. Ase, Ramin Raouf, Danny Bélanger, Edith Hamel, and Philippe Séguela
Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada

Received May 3, 2005; accepted June 13, 2005

ABSTRACT

Postsynaptic P2X1 ATP-gated channels are expressed in smooth muscle cells of the vascular and genitourinary systems, where they mediate desensitizing neurogenic contractions. Using the model of the isolated rat tail artery, we show that the vasoactive mediator 5-hydroxytryptamine (5-HT), via the 5-HT2A metabotropic receptor, regulates the desensitization kinetics of P2X1 responses by increasing their rate of recovery. Reconstituting the potentiation of P2X1 ATP-gated currents by 5-HT2A receptors in the Xenopus oocyte expression system, we provide evidence that this modulation depends on the activation of novel protein kinase C isoforms and protein kinase D (also named PKCδ) downstream of phospholipase Cβ. Other major kinases like Ca2+/calmodulin kinase II, protein kinase A, mitogen-activated protein kinases, and tyrosine kinases were found not to be involved. Moreover, we report that buffering intracellular Ca2+ ions with the chelator 1,2-bis(2-amino phosphonyloxyethyl)-4-amino-3-methyl-5-oxo-5-carbazole (BAPTA) decreases the rate of recovery of P2X1 responses and increases their sensitivity to potentiation by 5-HT2A receptors or by the diacylglycerol analog phorbol ester 12-myristate 13-acetate. We conclude that intracellular Ca2+ and a subset of diacylglycerol-dependent protein kinases regulate the activity of P2X1 receptor channels by modulating their recovery from desensitization.

Extracellular ATP depolarizes purinoceptive target cells within a few milliseconds by opening P2X ATP-gated cation channels. The P2X receptor channel gene family comprises seven subunits (P2X1–7) that associate to form homomeric and heteromeric ATP receptors (Torres et al., 1999; Khakh, 2001). P2X receptors are involved in diverse physiological functions such as pain transmission, immune response, and control of smooth muscle contraction (Burnstock, 2002; North, 2002). However, only fragmentary information is currently available on mechanisms of modulation of this class of receptor channels. The kinetics of desensitization of P2X2 is regulated by activation of protein kinase A (PKA) (Chow and Wang, 1998) and protein kinase C (PKC) (Boué-Grabot et al., 2000). Homomeric P2X3 and heteromeric P2X2+3 receptor channels are subject to regulation by G protein-coupled receptors and intracellular messengers because activation of the bradykinin B2 and substance P NK1 receptors and phorbol ester treatment have been shown to potentiate their responses in Xenopus oocytes (Paukert et al., 2001). In rat primary sensory neurons, Ca2+/calmodulin kinase II (CaMK II) up-regulates P2X3 receptor currents by promoting their trafficking to the cell surface (Xu and Huang, 2004). ATP-gated P2X1 channels are expressed in vascular and genitourinary smooth muscles, where they mediate a significant component of neurogenic contractions. During agonist application, the homomeric P2X1 receptor subtype desensitizes with fast kinetics and recovers only partially from desensitization after several minutes in heterologous expression systems (Torres et al., 1998; Lé et al., 1999) and in smooth

This work was possible with the financial support of Canadian Institutes of Health Research (E.H. and P.S.). P.S. is a Killam Scholar.

Abbreviations
PKA, protein kinase A; PKC, protein kinase C; CaMK II, Ca2+/calmodulin kinase II; 5-HT, 5-hydroxytryptamine; PLC, phospholipase C; PKD, protein kinase D; α,β-meATP, α,β-methylene ATP; U-73122, 1-[6-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.105.089045.

The Journal of Pharmacology and Experimental Therapeutics
Vol. 315, No. 1
0022-3565/05/3151-144–154$20.00
Copyright © 2005 by The American Society for Pharmacology and Experimental Therapeutics
JPET 315:144–154, 2005
89045/3048677
89045/3048677
muscle cell preparations (Ennion and Evans, 2001). Modulation of the desensitization of P2X1 responses by metabotropic glutamate receptor 1 was reported in the Xenopus oocyte heterologous system (Vial et al., 2004), and accelerated recovery of P2X1 responses by α₁-adrenergic receptors was observed in the vas deferens of guinea pig (Smith and Burnstock, 2004).

Evidence of metabotropic regulation of vascular P2X1 receptors came from studies in rabbit ear arteries, where fast purinergic responses were found potentiated following endothelin-1 receptor stimulation (La and Rand, 1993); however, no information was available on the intracellular pathways involved. The isolated smooth muscle of rat tail artery has been widely used to study sympathetic control of vascular tone (Bradley et al., 2003), and it displays a high density of P2X1 receptors (Bo and Burnstock, 1990; Vulchanova et al., 1996). Furthermore, the vasoactive mediator 5-hydroxytryptamine (5-HT) induces contractions in rat tail artery through activation of 5-HT₂A receptors (Watts, 1996; Froldi et al., 2003) linked to Gq and phospholipase C (PLC) and enhances contractile responses to other agonists (Van Nueten et al., 1981). We report here for the first time a strong modulation of the desensitization of arterial P2X1 responses following 5-HT₂A receptor activation. Using the Xenopus oocyte heterologous expression system as an experimental model for investigating the functional and physiologically relevant interactions between 5-HT₂A and P2X1 receptors, we identified converging intracellular mechanisms responsible for the modulation of P2X1 responses. We provide evidence that 5-HT-mediated P2X1 potentiation through activation of 5-HT₂A receptors involves both novel PKC isoforms and protein kinase D (PKD) and depends on the levels of intracellular Ca²⁺ ions.

Materials and Methods

Vascular Reactivity in Isolated Rat Tail Artery. Rats were sacrificed by decapitation under deep anesthesia in accordance with

![Figure 1](https://example.com/fig1.png)
approved guidelines of the McGill University Animal Care Committee. The tail arteries were removed and placed in cold Krebs-Ringer buffer (pH 7.4, 118 mM NaCl, 4.5 mM KCl, 1.0 mM MgSO4, 1.0 mM KH2PO4, 25 mM NaHCO3, 2.5 mM CaCl2, and 6.0 mM glucose) and freed from surrounding tissues under a dissecting microscope. They were cut into 2-mm-long segments and mounted on an L-shaped wire attached to a force-displacement transducer in an organ bath and connected to a polygraph. The arteries were maintained at 37°C and oxygenated with a mixture of 95% O2/5% CO2. After proper calibration of the polygraph and equilibration of the rat tail artery segments, vessels were tested for maximal contractile capacity with a Krebs′ solution supplemented with high K+ concentration (127 mM) and allowed to recover for an additional 45-min period (for details, see Bouchelet et al., 2000).

Vessels were submitted to two different experimental protocols: 1) α,β-methylene ATP (α,β-meATP, 10 μM) was added directly to vessel segments at basal tone for three consecutive applications with 3-min intervals between each application; and 2) vessel segments were preconstricted with 5-HT (30–300 nM) to obtain a small contractile response (20% of the K+ response). When the 5-HT-induced contraction was stable, 10 μM α,β-meATP was added, and its contractile response was measured. For all the experiments, this protocol was performed three times at 3-min intervals with rinses in fresh Krebs′ solution and recovery to basal tone between each application.

**Recombinant P2X Receptor Expression and Electrophysiology in Xenopus Oocytes.** Ovary lobes were surgically removed from *Xenopus laevis* frogs deeply anesthetized by immersion in Tricaine (0.2%; Sigma-Aldrich, St. Louis, MO). After treatment with type I collagenase in calcium-free OR2 solution for 2 h at room temperature, stage V–VI oocytes were manually defolliculated. Wild-type rat P2X1 subunit and 5-HT2A receptor cRNA were synthesized in vitro using the mMessage mMachine kit (Ambion, Austin, TX) from pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA) and then microinjected into the cytoplasm of oocytes. In coexpression experiments, 25 ng of P2X1 and 25 ng of 5-HT2A cRNA were injected in each oocyte. Oocytes were kept at 19°C in Barth′s solution containing 50 μg/ml of gentamycin and 1.8 mM CaCl2 for 48 h before recording.

Fig. 2. Activation of 5-HT2A receptors accelerates the recovery of P2X1 currents in *Xenopus* oocytes. A and B, representative ATP-evoked and 5-HT-evoked inward currents from oocytes coexpressing P2X1 and 5-HT2A receptors. C, time course of recovery of P2X1 responses normalized to the first response to ATP (n = 15). D, influence of the timing of 5-HT preapplications on the modulation of P2X1 responses normalized to the first response to ATP (n = 6). Gray bars represent P2X1 responses when 5-HT is applied for 1 min immediately after the third application of ATP. Black bars represent P2X1 responses when 5-HT is applied 1 min before the fourth application of ATP. ***, p < 0.001; **, p < 0.01; *, p < 0.05.
Two-electrode voltage-clamp recordings were performed at room temperature using an OC 725C amplifier (Warner Instruments, Hamden, CT) and glass pipettes (1–3 MΩ) filled with 3 M KCl. Oocytes were placed in a 500-μl recording chamber and were perfused at a flow rate of 10 to 12 ml/min with Ringer’s solution, pH 7.4, containing 115 mM NaCl, 5 mM NaOH, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES.

Unless specified, stimulations with 10 μM ATP (Sigma-Aldrich) dissolved in Ringer’s solution were applied five times with 5-min intervals. Because P2X1 responses were stable after the second application of ATP, 5-HT (1 μM) was bath applied for 5 min between the third and fourth ATP applications. Oocytes were injected (volume = 20 nl) and incubated for 1 h with the different drugs tested before recording. For all the calculations of final drug concentration, we estimated a cell volume of 2 μl. Potentiation index was defined as follows: fourth ATP-evoked P2X1 peak current/third ATP-evoked P2X1 peak current.

**Statistical Analysis.** All the values are expressed as mean ± S.E.M. Data were analyzed using Student’s t test or one-way analysis followed by Newman-Keuls multiple comparison test, with p < 0.05 taken as significant.

**Results**

**5-HT Accelerates Recovery of P2X1-Mediated Constrictive Responses in Rat Tail Artery.** Using the model of isolated rat tail artery, we confirmed that initial applications of the selective P2X agonist α,β-meATP (10 μM) to vessel segments led to strong muscle constrictions responses (Fig. 1A). Repeated applications of α,β-meATP induced decreasing responses, measured as maximal amplitudes of contractions (Fig. 1C), a distinctive behavior of desensitizing P2X1 receptors. Induction of small and stable preconstrictions of the vessel segments with 5-HT significantly enhanced the recovery of subsequent α,β-meATP-induced contractile responses (Fig. 1, B and C).

**Reconstitution of 5-HT₂A Receptor-Mediated Regulation of P2X1 Responses in Xenopus Oocytes.** Recombinant P2X1 receptors showed decreased responses following consecutive applications of 10 μM ATP (5 min apart) in the Xenopus oocyte heterologous expression system (Fig. 2, A and C). P2X1-desensitizing responses varied approximately between 20 and 50% of initial response and reached a steady-state response following the second application of ATP. Oocytes coexpressing P2X1 and 5-HT₂A receptors were exposed to 1 μM 5-HT for 5 min between the third and fourth ATP applications. Under these conditions, 5-HT transiently elicited inward currents (Fig. 2B), sometimes oscillatory in nature (see subsequent figures), corresponding to secondary activation of Ca²⁺-dependent chloride currents. Application of 5-HT significantly accelerated recovery of subsequent P2X1 response, as shown in Fig. 2, B and C. 5-HT₂A-mediated P2X1 potentiation was robust; however, it showed batch-to-batch variations with a potentiation index ranging from 1.5 to 2.4. We observed that application of 1 μM 5-HT for 10 s was sufficient to modulate P2X1 responses significantly (data not shown). Because metabotropic receptors are linked to second messenger cascades, we also examined the time course of P2X1 recovery following 5-HT₂A activation. In our experimental conditions, 5-HT needed more than 1 min to generate a measurable effect on P2X1 recovery. As shown in Fig. 2D, when 5-HT was applied for 1 min immediately following the third ATP stimulation, significant P2X1 recovery was recorded on the fourth ATP stimulation (recovery = 47 versus 27% control; n = 6). However, when 1-min 5-HT application preceded the fourth ATP stimulation, no effect on P2X1 responses on the fourth ATP stimulation was recorded. P2X1 recovery instead became significant on the fifth ATP stimulation 5 min later (Fig. 2D) (57 versus 31% control; n = 6).

**5-HT₂A-Mediated Potentiation of P2X1 Responses Involves PLC-Coupled Signaling Cascades.** To validate Gq coupling of 5-HT₂A receptors and subsequent activation of PLCβ in modulating of P2X1 responses, the PLC inhibitor U-73122 was used. Uncoupling of 5-HT₂A receptors from PLCβ following bath application of 10 μM U-73122 blocked both 5-HT-evoked Ca²⁺-activated chloride currents and P2X1 potentiation in Xenopus oocytes with a potentiation index of 0.71 (n = 6) compared with 1.68 in control conditions (n = 8), as shown in Fig. 3.

**Pseudorotol Esters Mimic whereas Stauroporine Blocks 5-HT₂A-Mediated Potentiation of P2X1 Responses.** Given that activation of PLCβ via 5-HT₂A generates diacylglycerol from phosphatidylinositol 4,5-bisphosphate, the effect of the diacylglycerol analog pseudrotol ester 12-myristate 13-acetate (PMA) was assessed. Application of 100 nM PMA for 5 min, which by itself did not evoke Ca²⁺-activated chloride currents, significantly potentiated P2X1 responses (potentiation index = 1.62; n = 8), whereas the inactive enantiomer 4α-PMA had no effect (potentiation index = 0.98; n = 7) (Fig. 4, A and B). In addition, the microbial alkaloid staurosporine, a potent inhibitor of PKC isofoms and several other classes of protein kinases, was used to test whether 5-HT₂A-mediated recovery involves protein kinases downstream of PLCβ. Incubation and injection of oocytes with staurosporine (500 nM) blocked 5-HT-induced potentiation of P2X1 response (potentiation index = 1.08; p = 0.07;
n = 6) compared with vehicle-treated oocytes (potentiation index = 1.70; n = 9) (Fig. 4D), indicating that diacylglycerol-sensitive kinases contribute to the 5-HT2A-mediated modulation of P2X1 responses.

5-HT2A-Mediated Effects on P2X1 Involve Novel PKC and PKD but not Classical PKC. We tested the effect of two inhibitors of classical PKC isoforms, chelerythrine and peptide19–31, with similar inhibition profiles (chelerythrine also inhibits the novel isoform) on 5-HT-mediated potentiation of P2X1 response. Neither chelerythrine (10 μM) (potentiation index of 1.84, n = 4 versus 1.74 in vehicle-treated oocytes, n = 8) nor peptide19–31 (1 μM) (potentiation index of 1.87, n = 4 versus 2.25 in vehicle-treated oocytes, n = 6) had any effect on the modulation of P2X1 responses by 5-HT (Fig. 5, A–C).

Calphostin C also inhibits PKC activity, but it does so by interacting with the regulatory diacylglycerol-binding domain of classical and novel PKC isoforms. We observed that calphostin C (1 μM) reduced significantly 5-HT-mediated potentiation of P2X1 response as shown in Fig. 6, A and D, with a potentiation index of 1.43 (p < 0.05; n = 5) compared with 2.23 in vehicle-treated oocytes (n = 6). Oocytes were also treated with other compounds structurally related to staurosporine but with a different inhibition profile: the PKC and PKD (also named PKCμ) inhibitor G66976 and the broad-spectrum inhibitor K252a with high affinity for PKD. G66976 (200 nM) was able to block P2X1 modulation (Fig. 6, B and D) with a potentiation index of 1.36 (p < 0.05; n = 5) compared with 2.00 in vehicle-treated oocytes (n = 6). K252a (500 nM) also blocked the 5-HT2A-mediated modulation (Fig. 6, C and D) with a potentiation index decreased to 1.22 (p < 0.05; n = 4) compared with 2.04 in vehicle-treated oocytes (n = 6). These results point to a major role for novel PKC isoforms, as well as PKD, in the diacylglycerol-sensitive modulation of P2X1 receptors.

CaMK II, PKA, Mitogen-Activated Protein Kinases, and Tyrosine Kinases Are Not Involved. Many intracellular pathways are known to be directly or indirectly activated downstream of PKC or PKD; therefore, we also tested selective inhibitors of several major classes of protein kinases: CaMK II, PKA, mitogen-activated protein (MAP) kinases, and tyrosine kinases. Treatment of the oocytes with KN62 (10 μM), an inhibitor of CaMK II, did not block the 5-HT2A-mediated P2X1 potentiation (potentiation index = 1.99 versus 1.57 in control conditions; n = 5–7; p = 0.045). The compound H89 (1 μM), an inhibitor of PKA, did not block potentiation either (potentiation index = 2.05 versus 2.04 in control conditions; n = 5–7; p = 0.99). Neither inhibition of MAP kinases by the mitogen-activated extracellular signal-regulated kinase-activating kinase kinase (MEKK) inhibitor
U0126 (1 μM) (potentiation index = 2.10 versus 1.78 in control conditions; n = 4–6; p < 0.33) nor inhibition of tyrosine kinases with genistein (10 μM) (potentiation index = 1.74 versus 1.91 in control conditions; n = 4–9; p = 0.80) had any effect on P2X1 potentiation. We concluded that CaMK II, PKA, MAP kinases, or tyrosine kinases do not play a significant role in the modulation of P2X1 receptors by PLC-linked metabotropic receptors.

Buffering Intracellular Ca²⁺ Facilitates P2X1 Potentiation. Another consequence of PLCβ activation is the inositol 1,4,5-trisphosphate-dependent release of Ca²⁺ ions from intracellular stores. Therefore, we investigated the role of intracellular Ca²⁺ release on the modulation of P2X1 responses by 5-HT₂A receptors. To buffer intracellular Ca²⁺ released following 5-HT₂A activation, oocytes were injected with the chelator BAPTA (50 or 500 μM). Only at 500 μM, BAPTA was able to block 5-HT-evoked Ca²⁺-activated chloride currents (Fig. 7, A and B) and had an inhibitory effect by itself on the recovery of P2X1 response (Fig. 7C). Injection of 500 μM BAPTA potentiated the P2X1 modulation induced by 5-HT (Fig. 7, B and D), with a potentiation index of 3.96 (n = 5) compared with 1.60 in vehicle-treated oocytes (n = 6), showing a requirement of intracellular Ca²⁺ ions for P2X1 receptor recovery. The facilitatory effect of BAPTA on the potentiation logically reflected an inverse relationship between the initial Ca²⁺-dependent recovery of P2X1 receptors and their modulation.

Levels of Intracellular Ca²⁺ Control Metabotropic Modulation. In a small number of batches of oocytes, recovery was higher than usual before 5-HT application, and no significant increase in P2X1 potentiation was observed following 5-HT₂A receptor activation, despite large 5-HT₂A-mediated inward chloride currents (Fig. 8A). The same batches of oocytes that were not able to show modulation of P2X1 by 5-HT did not respond to modulation by PMA, as shown in Fig. 8B. To test the role of intracellular Ca²⁺ ions in this phenotype, we injected BAPTA in these oocytes and found that this treatment was able to restore the modulation by 5-HT₂A with a potentiation index of 2.16 (n = 6) compared with 1.20 in vehicle-treated oocytes (p < 0.05; n = 6) (Fig. 8, C and E). Lowering intracellular Ca²⁺ with BAPTA also restored the PMA-mediated facilitation of P2X1 recovery with a potentiation index of 2.09 (n = 5) compared with 1.12 in vehicle-treated oocytes (p > 0.05; n = 6) (Fig. 8, D and F). These results confirm that modulation of P2X1 responses by 5-HT or PMA depends on reduced recovery of P2X1 responses, corresponding to situations where levels of intracellular Ca²⁺ are low.
Discussion

A significant component of smooth muscle contraction is mediated by neurogenic ATP through activation of postsynaptic P2X1 receptor channels in blood vessels and in the genitourinary tract (Mulryan et al., 2000). The P2X1 subtype of ATP-gated channels displays strong desensitization following repeated stimulations (Torres et al., 1998). Complete recovery of the initial P2X1 response can take several minutes in native preparations (Ennion and Evans, 2001) and recombinant systems (Le ˆ et al., 1999). Therefore, understanding the modes of regulation of the desensitization kinetics of P2X1 receptors has direct relevance to smooth muscle physiology and pathophysiology. Using the model of isolated rat tail artery, we confirmed that contractile P2X responses can take several minutes in native preparations (Ennion and Evans, 2001) and recombinant systems (Lê et al., 1999). Therefore, understanding the modes of regulation of the desensitization kinetics of P2X1 receptors has direct relevance to smooth muscle physiology and pathophysiology. Using the model of isolated rat tail artery, we confirmed that contractile P2X responses strongly desensitized during repeated α,β-meATP applications. Sensitivity to the P2X-selective α,β-meATP is not restricted to the P2X1 subtype because the homomeric P2X3 and the heteromeric P2X2+3 and P2X1+5 subtypes also share similar EC50 values (1 μM) for this agonist (Lambrecht, 2000). Patch-clamp recordings on isolated smooth muscle cells of the rat tail artery showed that application of ATP induces a large, rapidly activating and rapidly inactivating inward current (Sneddon, 2000). The properties of P2X receptors in arterial smooth muscle, namely, high sensitivity to α,β-meATP, rapid desensitization, and slow recovery, correspond to the properties of P2X1 receptors. Moreover, although other P2X subunits may also be expressed (Nori et al., 1998), P2X1 is the predominant subunit in smooth muscle cells (Collo et al., 1996; Lewis and Evans, 2001). All this evidence supports the fact that α,β-meATP-induced contractions of the rat tail artery are mainly mediated by P2X1 receptors. The monoamine 5-HT also has vasoactive properties through 5-HT2A receptors expressed at the surface of arterial smooth muscle cells; therefore, we investigated the impact of the activation of a metabotropic 5-HT receptor on native P2X1 responses. Our results show that activation of vascular 5-HT2A receptors allows P2X1 responses to recover faster, without significant changes in the amplitude of the initial response.

We were able to reconstitute the modulation of P2X1 receptor recovery by 5-HT2A receptors in the Xenopus oocyte heterologous expression system, thus suggesting that ubiquitous intracellular mechanisms are involved. Blockade of 5-HT2A-mediated P2X1 recovery by the PLC inhibitor U-73122 confirmed 5-HT2A receptor coupling to Gαq proteins and activation of PLC, while eliminating the possibility that Gβγ subunits could play a direct role. We observed that 5-HT-mediated P2X1 recovery was mimicked by the diacyl-
glycerol analog PMA and blocked by the protein kinase inhibitor staurosporine. These findings suggested a major role for diacylglycerol-sensitive PKC activation in potentiating P2X1 responses. Interestingly, it was shown that staurosporine also inhibited metabotropic glutamate receptor 1-mediated P2X1 potentiation (Vial et al., 2004). However, the latter study did not identify the specific kinases involved because staurosporine is a broad-spectrum inhibitor (Hidaka and Kobayashi, 1992).

The PKC family comprises several phospholipid-dependent serine/threonine protein kinases that have been grouped in three categories according to their primary sequence and activation requirements (Parker and Murray-Rust, 2004). The classical protein kinase C (cPKC) isoforms are Ca\(^{2+}\)-dependent and are activated by diacylglycerol or phorbol esters. The novel protein kinase C (nPKC) isoforms are Ca\(^{2+}\)-independent but are activated by diacylglycerol or phorbol esters, whereas the atypical PKC isoforms are unresponsive to both Ca\(^{2+}\) and diacylglycerol or phorbol esters. In addition, another Ca\(^{2+}\)-independent but phorbol ester-responsive kinase, PKD (PKC\(\mu\)), has been identified. Although originally included in the nPKC family, PKD differs significantly in critical structural features from the other PKC isoforms (Johannes et al., 1994, 1995).

We observed that two different cPKC inhibitors, chelerythrine (Herbert et al., 1990) and peptide\(_{19-31}\) (House and Kemp, 1987), had no effect on 5-HT-mediated P2X1 potentiation, indicating no role for cPKC in this modulation. However, we found that calphostin C, an inhibitor of diacylglycerol-sensitive PKC isoforms, significantly reduced P2X1 potentiation. Collectively, our results show that nPKC isoforms are responsible for a significant component of the 5-HT-mediated P2X1 potentiation. Accordingly, calphostin C blockade of noradrenaline-mediated P2X1 potentiation through \(\alpha_1\)-receptors in the vas deferens of guinea pig (Smith and Burnstock, 2004) could be the result of nPKC inhibition and not cPKC inhibition. When we tested G6976, a selective cPKC and PKD inhibitor (Martiny-Baron et al., 1993), a significant reduction of 5-HT-mediated P2X1 modulation was observed. Because cPKC isoforms are not involved in P2X1 potentiation, PKD is an obvious candidate. Noticeably, the staurosporine-related kinase inhibitor K252a induced a complete blockade of 5-HT-mediated P2X1 potentiation. It has been shown that K252a and G6976 (also a staurosporine-related compound) are more potent PKD inhibitors than staurosporine (IC\(_{50}\) = 7, 20, and 40 nM, respectively) (Gschwendt et al., 1996). Taken together, these findings

---

Fig. 7. P2X1 potentiation is facilitated by buffering intracellular Ca\(^{2+}\). A and B, representative current traces showing the impact of buffering intracellular Ca\(^{2+}\) release with BAPTA (vehicle versus 500 \(\mu\)M) on 5-HT-evoked Ca\(^{2+}\)-activated chloride currents and on 5-HT-induced potentiation of P2X1 responses in Xenopus oocytes. C, buffering intracellular Ca\(^{2+}\) significantly reduced P2X1 recovery following second and third ATP applications (\(n = 11\)). D, quantitative data on the facilitatory effects of BAPTA (500 \(\mu\)M) on 5-HT-induced P2X1 potentiation (\(n = 6\)) illustrated by the amplitude of the fourth response (after 5-HT) normalized to the third ATP response (before 5-HT). * \(P < 0.05\); ** \(P < 0.01\).
suggest an important contribution of PKD, in addition to nPKC isoforms, in the modulation of P2X1 responses by 5-HT2A receptors. We could rule out the contribution of other protein kinases potentially linked to 5-HT2A receptor activation (reviewed by Raymond et al., 2001), such as CaMK II, tyrosine kinases, PKA, and MAP kinases, because their selective inhibitors did not have any effect on the 5-HT2A-mediated modulation of P2X1 responses.

The other intracellular signal generated by 5-HT2A receptor transduction is the release of Ca\(^{2+}\) from intracellular stores. Buffering intracellular Ca\(^{2+}\) with 500 μM BAPTA restores full potentiation of P2X1 responses by 5-HT or PMA. E and F, quantitative effects of BAPTA treatment on 5-HT-induced (n = 6) or PMA-induced (n = 5) potentiation of the fourth P2X1 response (after 5-HT or PMA) normalized to the third response (before 5-HT or PMA). *p < 0.05.

Fig. 8. Buffering intracellular calcium can restore 5-HT- and PMA-mediated P2X1 potentiation. A and C, representative current traces showing small effects of 5-HT or PMA on P2X1 potentiation in some batches of oocytes despite high levels of 5-HT2A receptor expression. B and D, buffering intracellular Ca\(^{2+}\) with 500 μM BAPTA restores full potentiation of P2X1 responses by 5-HT or PMA. E and F, quantitative effects of BAPTA treatment on 5-HT-induced (n = 6) or PMA-induced (n = 5) potentiation of the fourth P2X1 response (after 5-HT or PMA) normalized to the third response (before 5-HT or PMA). *p < 0.05.
facilitate the recovery of ATP responses by promoting the phosphorylation of components of the P2X1 receptor channel complex, therefore limiting the possibilities of further potentiation by metabotropic receptors through nPKC/PKD phosphorylation.

The potentiation of P2X1 responses is mimicked by diacylglycerol analogs, mediated by nPKC and PKD activity, and facilitated by buffering intracellular Ca\(^{2+}\)\). Thus, agonist-induced diacylglycerol production and resting intracellular Ca\(^{2+}\) have converging modulatory effects on P2X1 receptors. Because it has been shown that phospholipase A2 (Paukert et al., 2001) and CaMKII (Xu and Huang, 2004) modulate the P2X3 receptor, potentiating effects of diacylglycerol and intracellular Ca\(^{2+}\) on other neuronal and non-neuronal P2X receptor subtypes endowed with different desensitization properties will remain to be explored.

Our findings show serotonergic modulation of vascular P2X1 receptor response through 5-HT\(_{2A}\) activation in a physiologically relevant functional interaction. This metabotropic modulation leads to faster recovery of desensitizing ATP responses in a smooth muscle preparation and in Xenopus oocytes. The level of extracellular ATP is activity-regulated and tissue-specific and is influenced by a variety of factors such as extracellular space, rate of ATP release, and hydrolysis (reviewed by Lazarowski et al., 2003). Any significant increase in ATP concentration linked to presynaptic activity may induce desensitization of postsynaptic P2X1-mediated ATP responses. We have observed that 5-HT\(_{2A}\) receptors are more efficacious in modulating P2X1 receptors when they display stronger desensitizing responses. The present study reports for the first time an activity-dependent cross talk between the two vasoactive neurotransmitters 5-HT and ATP, where the extent of P2X1 desensitization and the levels of postsynaptic intracellular Ca\(^{2+}\) may determine the effectiveness of 5-HT to potentiate ATP signaling. This may provide a mechanism to restrict the potentiating role of 5-HT on the amplitude of ATP-induced contractions to physiological situations where the levels of postsynaptic Ca\(^{2+}\) are low, i.e., during periods of low neuromuscular activity.

Other transmitters such as endothelin (La and Rand, 1993), histamine (Eto et al., 2001; Smith and Burnstock, 2004), and norepinephrine (Smith and Burnstock, 2004), through their respective metabolotropic receptor coupled to PLC, have also been shown to modulate ATP responses in other smooth muscle preparations. Therefore, dysregulations of diacylglycerol-sensitive pathways or intracellular Ca\(^{2+}\) levels may disrupt cross talk between metabolotropic and ionotropic contractile receptors, with potential pathological consequences on vascular tone or genitourinary tract function.

Acknowledgments

We thank Dominique Blais, Marie-France Witty, and Nella Sercu for expert technical assistance.

References


Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, and...


Address correspondence to: Dr. Philippe Séguela, Montreal Neurological Institute, 3801 University, Office 778, Montreal, QC, Canada H3A 2B4. E-mail: philippe.seguela@mcgill.ca