Local elementary purinergic-induced Ca\textsuperscript{2+} transients: from optical mapping of nerve activity to local Ca\textsuperscript{2+} signaling networks

David C. Hill-Eubanks, Matthias E. Werner, and Mark T. Nelson

1Department of Pharmacology, University of Vermont, Burlington, VT 05405
2Division of Cardiovascular and Endocrine Sciences, University of Manchester, Manchester M13 9PL, England, UK

The autonomic nervous system regulates smooth muscle contractility through both sympathetic and parasympathetic influences. In some tissues, such as the urinary bladder, parasympathetic influences predominate and nerves communicate to detrusor smooth muscle through the release of acetylcholine (ACh). In other tissues, such as the vas deferens and mesenteric arterial circulation, the primary autonomic influence is sympathetic, and norepinephrine (NE) is the predominant neurotransmitter. NE and ACh act on smooth muscle cells through G\textsubscript{\alpha}-coupled \(\alpha\)-adrenergic and muscarinic receptors, respectively, which signal through PLC to elevate diacylglycerol and inositol trisphosphate (IP\textsubscript{3}), which in turn, activate PKC and IP\textsubscript{3} receptors (IP\textsubscript{3}R) in the SR. IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from the SR of vascular smooth muscle cells gives rise to Ca\textsuperscript{2+} waves (Iino and Tsukioka, 1994; Jaggar and Nelson, 2000; Wray et al., 2005; Kim et al., 2008), which are propagating elevations in Ca\textsuperscript{2+} that are thought to contribute to vascular smooth muscle contraction (Mauban et al., 2001; Zang et al., 2006). The consequences of G protein–coupled signaling events manifest after a characteristic lag, reflecting the temporal dynamics of multiple sequential and parallel molecular linkages.

Although NE and ACh are the prototypical transmitters released by autonomic nerves, it has long been known that ATP is coreleased with NE at sympathetic nerve–muscular junctions and with ACh at parasympathetic nerve–muscular junctions. Coreleased ATP acts on P2X receptor channels in the plasma membrane of smooth muscle cells. Because P2X receptors are ion channels, once activated, their effects are experienced almost immediately by the cell. This rapid time course is in contrast to the more delayed influence of the G protein–coupled adrenergic and muscarinic receptors.

P2X receptors represent a family of seven receptors (P2X<sub>1–7</sub>) that belong to the transmitter-gated ion channel superfamiliy, which also includes nicotinic-like receptors and glutamate-like receptors (for review see Khakh, 2001). Each P2X receptor subunit possesses intracellular N and C termini and two membrane-spanning domains linked by a large extracellular domain (for review see Khakh, 2001; North, 2002). P2X receptors are thought to consist of three subunits (Aschrafi et al., 2004), which is also the simplest stoichiometry among ionotropic receptors. At least three ATP molecules bind to the extracellular domain of P2X channels (Jiang et al., 2003). Upon binding ATP, P2X receptors undergo conformational changes that result in the opening of the pore within milliseconds, although the underlying molecular details have not yet been elucidated.

P2X receptors are nonselective cation channels that exhibit a permeability to Ca\textsuperscript{2+} approximately equal to that of sodium (Na\textsuperscript{+}) (Schneider et al., 1991). Thus, activation of P2X receptors by ATP released at nerve–muscle junctions causes a rapid local influx of Na\textsuperscript{+} and Ca\textsuperscript{2+} (Lamont and Wier, 2002; Lamont et al., 2006). Although most of the excitatory junction current (EJC) associated with P2X activation is carried by the more abundant (~70-fold) Na\textsuperscript{+} ions, the influx of Ca\textsuperscript{2+} is quite substantial. In fact, the fractional Ca\textsuperscript{2+} currents mediated by the rat (~12.4%) and human (~11%) P2X<sub>1</sub> isoforms are not significantly different from that of the NMDA channel (~14%) (Egan and Khakh, 2004), long considered the gold standard for high-level, ligand-gated Ca\textsuperscript{2+} entry. The current mediated by Na\textsuperscript{+} and Ca\textsuperscript{2+} influx creates an excitatory junction potential (EJP) that contributes directly to the increase in postjunctional excitability associated with autonomic stimulation.

The P2X<sub>1</sub> receptor is the predominant P2X receptor isof orm expressed in smooth muscle. It was originally cloned from the vas deferens (Valera et al., 1994), and immunocytochemical studies in mice have shown that
P2X₁ expression in the urinary bladder is restricted to detrusor smooth muscle (Vial and Evans, 2000). The most compelling evidence for the prominence of the P2X₁ isoform in smooth muscle comes from studies using P2X₁ receptor knockout (P2X₁-R-KO) mice. These studies have shown that ATP-evoked EJCs and EJPs are absent in the vas deferens from P2X₁-R-KO mice (Mulryan et al., 2000). Similarly, these mice lack nerve-evoked purinergic contractile responses in bladders (Vial and Evans, 2000) and mesenteric arteries (Vial and Evans, 2002; Lamont et al., 2006).

Nerve-evoked elementary purinergic Ca²⁺ transients: NCTs, jCaTs, and NEPCaTs

As first demonstrated by Brain et al. (2002), the postjunctional action of ATP can be detected optically in the form of discrete, focal Ca²⁺ increases in smooth muscle cells. Using confocal microscopy and a mouse vas deferens preparation in which both smooth muscle and nerve varicosities are loaded with the Ca²⁺ indicator dye Oregon Green 488 BAPTA-1, these authors found that nerve stimulation evokes intermittent Ca²⁺ transients at tightly clustered sites immediately adjacent to nerve varicosities. These events, termed neuroeffector Ca²⁺ transients (NCTs), are temporally linked to the stimulating impulse (average delay, ~6 ms) and are preceded by an increase in Ca²⁺ in the adjacent nerve varicosity. NCTs are abolished by persistent exposure to the P2X agonist/desensitizing agent α,β-methylene ATP (α,β-meATP) and unaffected by inhibition of voltage-dependent Ca²⁺ channels (VDCCs), α₁-adrenergic receptors, or IP₃Rs (Brain et al., 2003), establishing their likely identity as Ca²⁺ influx mediated by ATP-activated P2X receptors.

Shortly thereafter, Wier and colleagues reported similar spatially localized Ca²⁺ transients in vascular smooth muscle cells of pressurized mesenteric arteries (Lamont and Wier, 2002). The authors termed these events junctional Ca²⁺ transients (jCaTs). Using the Ca²⁺-binding dye fluo-4 and fluorescence confocal microscopy, these authors showed that jCaTs are largely unaffected by ryanodine, which abolishes RYR-mediated Ca²⁺ sparks. Instead, they are blocked by the nonselective P2X receptor antagonist suramin, transiently induced by the application of the P2X receptor agonist (desensitizing agent) α,β-meATP, and absent in P2X₁-R-KO mice, confirming that these events are records of Ca²⁺ influx through postjunctional P2X₁ receptors (Lamont and Wier, 2002; Lamont et al., 2006). jCaTs induced by electrical field stimulation of associated nerves exhibit a close temporal relationship to the stimulus (latency, generally <3 ms). jCaTs also occur spontaneously, reflecting spontaneous neurotransmitter release.

Work in our laboratory has shown that both spontaneous and nerve-evoked elementary purinergic Ca²⁺ transients (NEPCaTs) can also been detected in the urinary bladder. These events are blocked by suramin and desensitization with α,β-meATP in rat urinary bladder smooth muscle, and are absent in P2X₁R-KO mice (Heppner et al., 2009), showing that they reflect Ca²⁺ entry through P2X₁ receptor channels. They are also unaffected by inhibitors of IP₃Rs (2-APB), RYRs (ryanodine), or VDCCs (dihydropyridines) (Heppner et al., 2005), confirming that they are distinct from Ca²⁺ “puffs,” Ca²⁺ sparks (Pérez et al., 1999; Jaggar et al., 2000), and the VDCC-mediated Ca²⁺ sparklets described by Santana and Navedo (2009) (see also Table I). In mouse bladders, spontaneous Ca²⁺ transients are coincident with spontaneous EJPs, and their magnitudes are correlated (Young et al., 2008), clearly linking these optical events with long-studied, postjunctional electrical events.

The kinetic properties of purinergic Ca²⁺ transients identified in vas deferens (NCTs), mesenteric arteries (jCaTs), and urinary bladder (NEPCaTs) are similar to

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vas deferens (NCT)¹</th>
<th>Mesenteric artery (jCaT)²</th>
<th>Urinary bladder (NEPCaT)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (t₁/₂) (ms)</td>
<td>120⁴; 280⁵</td>
<td>~145</td>
<td>~112</td>
</tr>
<tr>
<td>Area (µm²)</td>
<td>~12</td>
<td>~25</td>
<td>~14</td>
</tr>
<tr>
<td>Amplitude (F/F₀)</td>
<td>n/d</td>
<td>~2.8</td>
<td>~2.0</td>
</tr>
<tr>
<td>Amplitude (nM)</td>
<td>n/d</td>
<td>n/a</td>
<td>100–200</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>~6</td>
<td>~3</td>
<td>8–16</td>
</tr>
</tbody>
</table>

¹Brain et al., 2002.
²Lamont and Wier, 2002.
³Heppner et al., 2005.
⁴Perez et al., 1999.
⁵Jaggar et al., 2000.
⁶Ledoux et al., 2008.
⁷Santana and Navedo, 2009.
⁸Line scan.
⁹xy scan.
one another and are clearly distinct from those of other focal Ca^{2+} transients (Table I). The spatial spread and duration ($t_{1/2}$) of these events in the bladder are 14 µm$^2$ and $\sim$112 ms, respectively, and the corresponding values for mesenteric artery jCaTs are $\sim$20 µm$^2$ and 145 ms. Using line scanning to analyze the kinetics of NCTs, Brain et al. (2002) showed that NCTs measured in mouse vas deferens have a spatial spread of $\sim$12 µm$^2$ and decay with a first-order time constant ($t_{1/2}$) of $\sim$120 ms. The decay time constant obtained by xy scanning is much larger ($\sim$280 ms), a difference that was attributed to the contribution of cytoplasmic diffusion of Ca^{2+} near the site of entry.

The kinetic properties of spontaneous and evoked purinergic transients are the same, suggesting that these events are caused by the quantal release of ATP. This is consistent with earlier evidence that quantal release of ATP is responsible for EJPs and/or EJCs in femoral and mesenteric arteries, rat tail artery, and vas deferens (for review see Stjärne and Stjärne, 1995). The low probability, highly intermittent quality of the events recorded in these electrophysiological studies conforms well with the predictions of the “intermittent model” developed to describe NE release from sympathetic nerves, which posits that a single vesicle in $\sim$1% of all varicosities releases its entire content in response to a nerve impulse (Stjärne and Stjärne, 1995, and references therein).

Exploiting this logic, Cunnane and colleagues have used NCTs as a means to detect “packeted release” of ATP from nerve terminals (Brain et al., 2002; Young et al., 2007; Brain, 2009). Their results based on electrophysiological measurements in single smooth muscle cells showed that the amplitude distribution of spontaneous EJPs is skewed, suggesting a broad distribution of spontaneously released neurotransmitter packet size (Young et al., 2007). Although bulk changes in ATP release can be monitored electrophysiologically as EJPs (or EJCs), this approach is less suitable for mapping quantal transmitter release because smooth muscle cells are large and electrically coupled, making it difficult to determine whether the recorded event originates in the cell being recorded (and if so, where), or is caused by release events that occur at some distance removed from the recording site. Optically measuring Ca^{2+}, released focally by ATP-activated P2X$_1$Rs, overcomes these limitations, allowing more accurate spatial mapping of ATP release sites. And because ATP-induced, P2X receptor–mediated Ca^{2+} influx is very rapid, optical mapping also provides fine temporal resolution of the underlying transmitter release events. jCaTs in mesenteric arteries (Lamont and Wier, 2002) and NEPCaTs in urinary bladder (Heppner et al., 2005) have also been used to optically map ATP release by sympathetic and parasympathetic nerves, respectively.

In addition to spatial and temporal mapping of ATP release events, NCTs can provide information about coreleased transmitters and their potential local modulation of transmitter release probability. One example of this is using NCTs to monitor the prejunctional autoinhibitory effects of neurally released transmitters. In this context, the frequency of nerve-evoked NCTs was...
shown to increase in the presence of the α2-adrenoceptor inhibitor yohimbine (Brain et al., 2002), providing evidence that coreleased NE acts through prejunctional α2-adrenoceptors to reduce nerve terminal Ca2+ concentration and decrease the probability of exocytosis (Brain et al., 2002; Brain, 2009). In a similar vein, potential off-target effects of pharmacological agents on prejunctional targets can be inferred from changes in the frequency of purinergic Ca2+ transients upon the application of such agents, a strategy we have used in studies on the urinary bladder and mesenteric arteries (unpublished data).

To the extent that release of different transmitters is coupled (i.e., not differentially regulated), detection of local purinergic Ca2+ transients could provide the means to optically map nerve activity generally. Whether ATP and NE in sympathetic nerve terminals are stored and/or released together has been extensively studied by Stjärne and colleagues. This seemingly straightforward question is deceptively difficult to answer, especially given the available experimental tools. Early reports from this group based on electrochemical and electrophysiological studies in rat tail arteries suggested that ATP and NE are indeed released in parallel by nerve stimulation, with apparent deviations from this conclusion likely reflecting differences in clearance rates (for review see Stjärne and Stjärne, 1995). The use of a paired-pulse stimulus paradigm provided support for this interpretation, showing that the dramatic depolarization of paired-pulse transmitter release caused by K+ channel block has similar effects on NE oxidation currents and ATP-mediated EJCs, and purinergic and adrenergic contractile responses (Mshghina et al., 1998). However, more recent work by these researchers presents a more nuanced picture. The results of these studies suggest that ATP and NE are stored in separate small vesicles that are released in parallel upon low frequency stimulation (<2 Hz), but above this frequency show increasingly nonparallel release (Stjärne, 2001).

Implications of local Ca2+ signaling: Ca2+-signaling networks

Individual purinergic Ca2+ transients have the potential to signal locally to modulate Ca2+-sensitive processes (Fig. 1). Although this is largely unexplored territory, some features of such local signaling networks can be discerned from published reports, and it is possible to speculate about others.

Action potential trigger or current injection. A single purinergic Ca2+ transient represents the activation of a cluster of P2X,Rs by local ATP from a nerve varicosity. This local injection of current could conceivably trigger an action potential. Indeed, Young et al. (2008) recently demonstrated that in intact UBSM strips from mice, single NCTs cause spontaneous depolarizations (“sDep’s”) and can trigger “spontaneous” action potentials. These action potentials cause phasic contractions, which contribute to muscle tone during bladder filling; an increase in their activity is a hallmark of unstable detrusor and urinary bladder dysfunction. The observation that purinergic Ca2+ transients can trigger action potentials suggests that bladder filling is also under local neurogenic control.

In vas deferens, NCTs do not map to action potentials in a simple one-to-one relationship (Brain et al., 2002). Although not all NCTs elicit an action potential, ~20% of NCTs are rapidly (~0.5 s) followed by an action potential. Consistent with this, purinergic Ca2+ transients in bladder co-occur with large increases in global Ca2+ termed “flashes” (Heppner et al., 2005). Both purinergic Ca2+ transients and Ca2+ flashes occur spontaneously, and the frequency of both types of events is increased by nerve stimulation. Ca2+ flashes are associated with tissue contraction and are eliminated by dihydropyridines, indicating that they are caused by Ca2+ influx through VDCCs during an action potential. Purinergic Ca2+ transients are unaffected by inhibition of VDCCs, but inhibition of P2X receptors abrogates Ca2+ flashes, implying that the cationic flux registered by the optical NCT/jCaT/NEPCaTs compared with sparks. Ryanodine reduces jCaT amplitude by a much more modest, but Ca2+ influx through a cluster of P2X,Rs may activate nearby RYRs in the SR, which should contribute to the purinergic Ca2+ transient (Fig. 1). In vas deferens, unlike mesenteric artery and urinary bladder smooth muscle, inhibition of RYRs with ryanodine substantially reduces the amplitude of NCTs (~45%), and activation of RYRs with caffeine (3 mM) induces a dramatic (16-fold) increase in the frequency of NCTs (Brain et al., 2003). In addition, the inhibition of SR Ca2+ uptake by Ca2+ SR/ER-ATPase with cyclopiazonic acid increases the half-life of these events. These results suggest a functional unit in which Ca2+ influx mediated by P2X1 stimulates CICR from RYRs, which augments the local P2X1-mediated Ca2+ signal. According to the model proposed by Brain et al. (2003), the duration of the Ca2+ signal is governed by the summation of these two Ca2+ release events as well as the rate at which released Ca2+ is sequestered by the SR through Ca2+ SR/ER-ATPase pump activity. Such a mechanism would be consistent with the larger spread and longer half-life of NCT/jCaT/NEPCaTs compared with sparks. Ryanodine reduces jCaT amplitude by a much more modest, but
significant, ~13% in mesenteric arteries (Lamont and Wier, 2002), and does not appear to affect purinergic Ca$^{2+}$ transients in the urinary bladder (Heppner et al., 2005), suggesting that the effect of RYR inhibition is at least quantitatively different among these different smooth muscle tissues. Consistent with the lack of CICR in the regulation of spontaneous phasic contractions, the inhibition of RYRs does not decrease, but instead enhances, the frequency of phasic contractions in the urinary bladder smooth muscle from the guinea pig (Herrera et al., 2000). Depending on the relative speed of IP$_3$ production by concurrent activation of adrenergic or muscarinic receptors, it is also possible that Ca$^{2+}$ influx through P2X$_1$Rs could amplify local IP$_3$R activation by influx of Ca$^{2+}$ associated with a purinergic Ca$^{2+}$ transient that has not yet been experimentally tested is that the smooth muscle P2X receptors. 

consistent with a possible postjunctional influence of adrenergic signaling in this setting, receptors blocked with suramin (Rummery et al., 2007). presence of functional P2X receptors than it is with P2X adrenergic component is substantially higher in the constriction is similar in the presence and absence of the α$_1$-adrenergic receptor antagonist prazosin, but the adrenergic component is substantially higher in the presence of functional P2X receptors than it is with P2X receptors blocked with suramin (Rummery et al., 2007). Thus, it appears that purinergic activity may exert a potentiating effect on adrenergic signaling in this setting, consistent with a possible postjunctional influence of smooth muscle P2X receptors.

**NCT/JCaT/NEPCaT to K$_{Ca}$ channel communication.** The timing, nature, and proximity of the actions of coreleased ATP and NE/ACh suggest that local Ca$^{2+}$ influx through P2X$_1$ receptors might modulate the subsequent effects of nerve-evoked NE/ACh release on smooth muscle. Nerve-released ATP acts rapidly on postjunctional P2X$_1$Rs to cause a rapid influx of Ca$^{2+}$ (and Na$^+$), and the coreleased NE and ACh act more slowly on their respective G$_{	ext{a}}$-coupled receptors. One intriguing possibility that has not yet been experimentally tested is that the influx of Ca$^{2+}$ associated with a purinergic Ca$^{2+}$ transient might activate Ca$^{2+}$-sensitive K$^+$ (K$_{Ca}$) channels, such as small-conductance SK or large-conductance BK channels (Fig. 1). Such a mechanism might conceivably account for our observation that the cholinergic component of parasympathetic nerve-evoked action potentials in mouse urinary bladder is augmented by inhibition of P2X receptors (with suramin or α,β-meATP, or by genetic ablation of P2X$_1$Rs) (Heppner et al., 2009). These results imply that ATP-mediated P2X$_1$R activity normally exerts an inhibitory influence on the subsequent ACh–muscarinic receptor signaling pathway. If K$_{Ca}$ channels are, in fact, activated by purinergic Ca$^{2+}$ transients, their activity would be predicted to dampen cholinergic signaling and limit the duration of the cholinergic action potential through their membrane hyperpolarizing action.

Interestingly, purinergic signaling may have the opposite effect on NE signaling in mesenteric arteries, at least in fully pressurized arteries (90 mmHg). Here, the purinergic component of sympathetic nerve-evoked vasoconstriction is similar in the presence and absence of the α$_1$-adrenergic receptor antagonist prazosin, but the adrenergic component is substantially higher in the presence of functional P2X receptors than it is with P2X receptors blocked with suramin (Rummery et al., 2007). Thus, it appears that purinergic activity may exert a potentiating effect on adrenergic signaling in this setting, consistent with a possible postjunctional influence of smooth muscle P2X receptors.

**Closing thoughts.** Purinergic Ca$^{2+}$ transients, by whatever name, are likely a common feature of nerve–smooth muscle junctions, where they may feed into tissue-specific local Ca$^{2+}$ signaling networks and potentially modulate a myriad of Ca$^{2+}$-dependent processes. Most of the potential connections between NCT/JCaT/NEPCaT-like events and intracellular signal transduction have not yet been experimentally tested and remain a matter of conjecture. Regardless of the functional roles that these events prove to play in postsynaptic smooth muscle cells, they should provide a convenient and sensitive optical read-out of neurally released ATP specifically and, to an as-yet-undetermined extent, of neural activity generally.

This Perspectives series includes articles by Gordon, Parker and Smith, Xie et al., Prosser et al., and Santana and Navedo.

This work was supported by National Institutes of Health (grants R37DK 053832, RO1 DK065947, RO1 HL44455, PO1 HL077378, P20 RO16435, and RO1 HL098243); Totman Trust for Medical Research, Research into Ageing (grant P332); The Royal Society (grant RG080197); and the British Heart Foundation (grant PG/07/115).

**REFERENCES**


Hill-Eubanks et al. 153


