Ca\textsuperscript{2+}-sensing Transgenic Mice

POSTSYNAPTIC SIGNALING IN SMOOTH MUSCLE*

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Genetically encoded signaling proteins provide remarkable opportunities to design and target the expression of molecules that can be used to report critical cellular events in vivo, thereby markedly extending the scope and physiological relevance of studies of cell function. Here we report the development of a transgenic mouse expressing such a reporter and its use to examine postsynaptic calcium signaling in smooth muscle. The circularly permuted, Ca\textsuperscript{2+}-sensing molecule G-CaMP (Nakai, J., Ohkura, M., and Imoto, K. (2001) Nat. Biotechnol. 19, 137–141) was expressed in vascular and non-vascular smooth muscle and functioned as a lineage-specific intracellular Ca\textsuperscript{2+} reporter. Detrusor tissue from these mice was used to identify two separate types of postsynaptic Ca\textsuperscript{2+} signals, mediated by distinct neurotransmitters. Intrinsic nerve stimulation evoked rapid, whole-cell Ca\textsuperscript{2+} transients, or “Ca\textsuperscript{2+} flashes,” and slowly propagating Ca\textsuperscript{2+} waves. We show that Ca\textsuperscript{2+} flashes occur through P2X receptor stimulation and ryanodine receptor-mediated Ca\textsuperscript{2+} release, whereas Ca\textsuperscript{2+} waves arise from muscarinic receptor stimulation and inositol trisphosphate-mediated Ca\textsuperscript{2+} release. The distinct ionicotropic and metabotropic postsynaptic Ca\textsuperscript{2+} signals are related at the level of Ca\textsuperscript{2+} release. Importantly, individual myocytes are capable of both postsynaptic responses, and a transition between Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and inositol trisphosphate waves occurs at higher synaptic inputs. Ca\textsuperscript{2+} signaling mice should provide significant advantages in the study of processive biological signaling.

Genetically encoded cell reporters of cell signaling hold great promise for the study of in vivo cell physiology. This field has advanced rapidly recently with the development of fluorescence resonance energy transfer-based probes such as cameleon (2), fluorescent proteins that can be modified following expression (3), and re-engineered fluorescent proteins (1, 4) (for review, see Ref. 5). The use of these tools as transgenes in mammals therefore has significant advantages in the study of processive biological signaling.

Genetically encoded signaling proteins provide remarkable opportunities to design and target the expression of molecules that can be used to report critical cellular events in vivo, thereby markedly extending the scope and physiological relevance of studies of cell function. Here we report the development of a transgenic mouse expressing such a reporter and its use to examine postsynaptic calcium signaling in smooth muscle. The circularly permuted, Ca\textsuperscript{2+}-sensing molecule G-CaMP (Nakai, J., Ohkura, M., and Imoto, K. (2001) Nat. Biotechnol. 19, 137–141) was expressed in vascular and non-vascular smooth muscle and functioned as a lineage-specific intracellular Ca\textsuperscript{2+} reporter. Detrusor tissue from these mice was used to identify two separate types of postsynaptic Ca\textsuperscript{2+} signals, mediated by distinct neurotransmitters. Intrinsic nerve stimulation evoked rapid, whole-cell Ca\textsuperscript{2+} transients, or “Ca\textsuperscript{2+} flashes,” and slowly propagating Ca\textsuperscript{2+} waves. We show that Ca\textsuperscript{2+} flashes occur through P2X receptor stimulation and ryanodine receptor-mediated Ca\textsuperscript{2+} release, whereas Ca\textsuperscript{2+} waves arise from muscarinic receptor stimulation and inositol trisphosphate-mediated Ca\textsuperscript{2+} release. The distinct ionicotropic and metabotropic postsynaptic Ca\textsuperscript{2+} signals are related at the level of Ca\textsuperscript{2+} release. Importantly, individual myocytes are capable of both postsynaptic responses, and a transition between Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release and inositol trisphosphate waves occurs at higher synaptic inputs. Ca\textsuperscript{2+} signaling mice should provide significant advantages in the study of processive biological signaling.

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2 The abbreviations used are: smMHC, smooth muscle isoform of myosin heavy chain; CICR, calcium-induced calcium release; GFPP, green fluorescent protein; αβ meATP, αβ methyleneATP; InsP\textsubscript{3}, inositol trisphosphate.
NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 glucose, 10 HEPES, pH 7.4).perfused with buffered extracellular solution consisting of (mM): 137
maintain physiological length of the tissue. The muscle segments were
containing a stimulating electrode and kevlar fibers placed on top to
face on the bottom of an optical recording chamber and a retaining clip
conduction pathways. The segments were placed with the serosal sur-
along the axis from the neck to the fundus were cut to preserve nerve
removed and dissected on ice. Segments of the urinary bladder running
moter construct consisted of
smMHC
pro-
ogram of transgene. The rat smooth mus-
natured eGFP (eGFPn and eGFPc, N and C-terminal fragments, respectively), and
calmodulin (CaM), was placed directly
downstream of a Kozak sequence (linkers
are indicated by red bars). Arrows show
the position of primers used for genotyping,
which are specific for circularly permutated eGFP. Unique restriction sites
are shown. B, genotyping. PCR
identifying the 423-bp G-CaMP fragment
in one founder (smGC-2). The PCR fragment
identifies a circularly permuted GFP sequence. C, transgene expression.
Sections from smGC3 mice were pro-
cessed with pre-immune (left) or anti-GFP
(right) antisera, demonstrating robust ex-
pression of the transgene confined to
smooth muscle tissues. Magnifications are
urinary bladder ×4, coronary artery
×40, bronchus ×40, and colon ×20.

bazoole substrate (Zymed Laboratories Inc. kit) for 5 min at room tem-
temperature. Sections were then washed and counterstained with
haematoxylin before mounting with fluoromount.

Imaging—smGC mice were euthanized with CO₂ and tissues rapidly
removed and dissected on ice. Segments of the urinary bladder running
along the axis from the neck to the fundus were cut to preserve nerve
conduction pathways. The segments were placed with the serosal sur-
facing the bottom of an optical recording chamber and a retaining clip
containing a stimulating electrode and kevlar fibers placed on top to
maintain physiological length of the tissue. The muscle segments were
perfused with buffered extracellular solution consisting of (mM): 137
NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 glucose, 10 HEPES, pH 7.4).
Stimulation pulses were generated by a Grass S48 stimulator con-
trolled by computer-generated TTL pulses and confocal images ob-
tained with a Bio-Rad Radiance 2000 confocal head attached to a Nikon
TMIII inverted microscope. Confocal x-y images were obtained at 71-ms
intervals and the stimulation signal encoded in the confocal image.
Transmitted light images indicated contractions in individual myo-
cytes, particularly during sustained or high frequency stimulations.
The effect of individual cell movement on fluorescence in the isometric
contractions were distinguished in consecutive images. Single cells were dissociated as previ-
ously described (8). Mesenteric artery images were recorded at 2
frames/s using a Nipkow spinning disk confocal head. Images showing
cross-sections of the arterial walls were captured through the center,
parallel to the long axis, of an intact pressurized (70 mm Hg) mesenteric
artery at 35 °C. The lung slice preparation was similar to that previ-
ously described (9). Briefly, the trachea was cannulated and the lungs
filled with 2% agarose (type VII-A: low gelling temperature) before
removal. Following cooling, single lobes were sectioned at 100 μm using a
vibrated tissue sectioner (OTS-5000; Electron Microscopy Sciences).
Confocal images were obtained using a Bio-Rad Radiance 2000 confocal
scan head coupled to a Nikon inverted microscope. In both cases, fluo-
rescence profiles were normalized by dividing the time-dependent flu-
orescence (F) by the average prestimulation fluorescence (F₀).

In Situ Calibration of G-CaMP—Individual muscle segments from
smGC mice were incubated in 100 μg/ml saponin in physiological solu-
tion at room temperature for 1 h. Segments were mounted on the
microscope and fluorescence measured at fixed excitation strength and
photomultiplier gain during exposure to solutions in which EGTA and
Ca²⁺ were added at mM concentrations to fix the [Ca²⁺]₀,res using the

![Diagram](image-url)
WINMX programs (10). Mean fluorescence was averaged for 3–5 myocytes within each image for each solution condition, these values were averaged, and the means of all experiments were fit by least squares to the Hill equation: 

\[ \frac{F - F_0}{(F_{\text{max}} - F_0)} = \frac{1}{1 + \left(\frac{x}{K_d}\right)^n} \]

where \( F \), \( F_0 \), and \( F_{\text{max}} \) are the observed, zero \( Ca^{2+} \), and saturating \( Ca^{2+} \) fluorescence, respectively, \( n \) the Hill coefficient, \( K_d \) the affinity constant, and \( x \) the pCa.

RESULTS

Production of Smooth Muscle G-CaMP Transgenic Mice—The \( Ca^{2+} \)-sensitive, circularly permuted eGFP variant G-CaMP (1) was used to construct a transgene by insertion downstream of the transcriptionally active first intron of the smMHC promoter (6, 11) (Fig. 1A), which we have previously used to direct robust, smooth muscle-specific eGFP expression in mice (7). The G-CaMP \( Ca^{2+} \) reporter utilizes a circularly permuted eGFP fusion protein that obtains a photoactive conformation upon binding of the C-terminal \( Ca^{2+} \)/calmodulin with the N-terminal M13 recognition peptide from myosin light chain kinase. Three founders were crossed with C57BL/6 mice and experiments conducted in two of these lines with the highest expression (smGC-2 and smGC-3, Fig. 1B). Hemizygous smGC mice produce litters of normal size and sex ratio and have expected longevity; histopathological analysis revealed no discernable phenotype associated with the expression of the \( Ca^{2+} \)-sensing reporter. Transgene expression was examined by immunostaining with an anti-eGFP polyclonal antibody that recognizes the circularly permuted G-CaMP. As shown in Fig. 1C, the transgene is robustly expressed in vascular and nonvascular smooth muscle and expression is confined to tissues of smooth muscle lineage. Importantly, expression is observed in virtually all cells within a muscle tissue, an obvious advantage in the context of examining cell signaling.

\( Ca^{2+} \)-sensing in Transgenic Mouse Cells—We examined the function of G-CaMP in a range of smooth muscle tissues. To confirm \( Ca^{2+} \) signaling under optimal optical conditions, G-CaMP was first examined in single myocytes dispersed from the urinary bladder of smGC mice. As shown in Fig. 2A, enzymatically dispersed myocytes display a low but detectable level of \( Ca^{2+} \) fluorescence (red circle) shows marked oscillatory responses. D, G-CaMP \( Ca^{2+} \) signaling in arterial vascular smooth muscle during vasoconstriction. Top, selected confocal images before and during application of 10 \( \mu \)M phenylephrine to an isolated, pressurized mesenteric artery. Bottom left, normalized arterial myocyte fluorescence from the entire image series. Bottom right, higher resolution confocal image of single layer of arterial myocytes during phenylephrine vasoconstriction. All calibration bars are 10 \( \mu \)m.
of fluorescence at rest and respond to Ca\(^{2+}\)-mobilizing stimuli (application of 100 \(\mu \text{M}\) methacholine) with a greater than 2-fold increase in fluorescence. The Ca\(^{2+}\) reporter is diffusely distributed in the cytosol with no evidence of organellar accumulation, in organelles, which is a common problem associated with membrane-permeant small molecule indicators, and the indicator reports typical Ca\(^{2+}\) oscillations and waves in myocytes despite the slower fluorescence kinetics than achieved, for example, with small organic molecules with low \(K_d\), such as Fluo-4 (1, 12).

A significant advantage of genetically encoded reporters is the ability to monitor intracellular signaling events in defined cell lineages within complex multicellular tissues without disrupting cell-cell contacts or permeabilizing membranes, a frequent necessity for efficient loading of small molecule reporters. Because G-CaMP does not retain fluorescence emission at 37 °C (1), experiments were conducted at room temperature in \textit{ex vivo} tissues. The Ca\(^{2+}\) dependence of G-CaMP fluorescence was calibrated in saponin-permeabilized urinary bladder muscle segments exposed to solutions with fixed free Ca\(^{2+}\) ranging from 50–1.8 nM and 1 \(\mu \text{M}\) Mg\(^{2+}\). As shown in Fig. 2B, in three such experiments the fluorescence of individual cells within muscle segments increased sharply over the physiological range of cellular free Ca\(^{2+}\). The fit of these data to the Hill equation yielded a \(K_d\) of 328 nM and a Hill coefficient of 2.9, which may be compared with the values of 235 nM and 3.3 for the recombinant protein measured \textit{in vitro} (1). There is a well documented increase in the apparent \(K_d\) of Ca\(^{2+}\) indicators measured in the presence of cellular proteins compared with \textit{in vitro} measurements in physiological salt solutions, probably due to substantial protein binding of the indicator molecules (13–15). Given the numerous cellular calmodulin-binding proteins and the effect of protein binding on the Ca\(^{2+}\) affinity of calmodulin (16), it is likely that interactions between G-CaMP and cellular proteins markedly influence the apparent affinity of the complex for Ca\(^{2+}\). The apparent \(K_d\) and Hill coefficient of G-CaMP in smGC mice indicate that ~93% of the total Ca\(^{2+}\)-dependent fluorescence occurs over the range between 100 nM and 1 \(\mu \text{M}\) free [Ca\(^{2+}\)]. Thus, G-CaMP is a useful tool for the measurement of cytosolic Ca\(^{2+}\) signaling, although the high Hill coefficient relative to small molecule dual wavelength (17) or single wavelength (12) Ca\(^{2+}\) indicators indicates that G-CaMP will be fully bound (and fluorescence thus saturate) under conditions of maximal Ca\(^{2+}\) signaling.

The function of G-CaMP was verified in lung, arterial, and urinary bladder smooth muscle. As shown in Fig. 2, Ca\(^{2+}\) signaling was observed in bronchial smooth muscle during constriction of a small airway produced by application of 50 \(\mu \text{M}\) methacholine in arterial myocytes during adrenergic vasoconstriction. Dynamic changes in [Ca\(^{2+}\)], in myocytes within these complex tissues could easily be resolved, and Ca\(^{2+}\) oscillations in individual myocytes were routinely observed. These measurements confirm the utility of smGC mice for the examination of dynamic Ca\(^{2+}\) signaling in a range of physiological preparations. Moreover, Ca\(^{2+}\) signaling can be measured in what is often a single layer of smooth muscle during physiolog-
ical contractions without disturbing the essential integrity of the tissues.

**Dual Modes of Postsynaptic Ca\(^{2+}\)/H11001 Signaling in Urinary Bladder Smooth Muscle**—Neuromuscular coupling in many smooth muscles, including the urinary bladder, is known to result from the co-release of acetylcholine and ATP (for review, see Ref. 18), although little is known about the nature of nerve-evoked Ca\(^{2+}\)/H11001 signals or the precise role of specific neurotransmitters in mediating distinct postsynaptic Ca\(^{2+}\)/H11001 events. We used smGC mice to examine postsynaptic neuromuscular signaling in the intact mouse detrusor (urinary bladder) by field-stimulating intrinsic nerves at a distance of several centimeters from imaged myocytes (Fig. 3A). All postsynaptic Ca\(^{2+}\)/H11001 signals were eliminated by application of 1 \(\mu\)M tetrodotoxin (data not shown), confirming that the responses consisted of conducted action potentials in intrinsic nerves. Low frequency (0.5–1.0 Hz) stimulation evoked random Ca\(^{2+}\) responses in individual myocytes (Fig. 3B) rather than synchronous responses in all cells, which likely reflects the low probability of neurotransmitter release at individual synapses (19). Postsynaptic Ca\(^{2+}\)/H11001 responses resulted in 1.5–3-fold increases in fluorescence of the genetically encoded reporter in situ, similar to the responses observed in single cells (Fig. 2).

Interestingly, two distinct postsynaptic Ca\(^{2+}\)/H11001 transients were observed. The most common events, which we term Ca\(^{2+}\)/H11001 flashes, were transient increases in \([Ca^{2+}];\) confined to individual myocytes, or occasionally 2–3 myocytes, characterized by a rapid, almost simultaneous rise in \([Ca^{2+}];\) throughout the entire myocyte (Fig. 3B). This mode of Ca\(^{2+}\)/H11001 signaling is quite similar to spontaneous events reported in arterial smooth muscle loaded with fluo-3 (20). Less frequently, a second pattern of Ca\(^{2+}\)/H11001 firing was observed, consisting of slowly propagating, asynchronous Ca\(^{2+}\)/H11001 waves. Asynchronous Ca\(^{2+}\)/H11001 waves dominated the postsynaptic response at higher stimulation frequencies (4–10 Hz), suggesting different thresholds for the modes of Ca\(^{2+}\)/H11001 signaling. Examples of these markedly different postsyn-

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**Fig. 5. Mechanism of Ca\(^{2+}\)/H11001 release in postsynaptic Ca\(^{2+}\)/H11001 flashes and Ca\(^{2+}\)/H11001 waves.** A, distinct properties of Ca\(^{2+}\)/H11001 flashes and Ca\(^{2+}\)/H11001 waves. Mean amplitude (\(F/F_0\)) and kinetics (\(s\)) for Ca\(^{2+}\)/H11001 flashes and waves and frequency of events before and after exposure to 1 \(\mu\)M atropine. B, Ca\(^{2+}\)/H11001 flashes are not associated with cholinergic neurotransmission. Mean Ca\(^{2+}\)/H11001 fluorescence within a single myocyte undergoing repeated nerve stimulation (arrow) demonstrates virtually identical postsynaptic Ca\(^{2+}\)/H11001 flashes in the same cell before and after application of 1 \(\mu\)M atropine. Following perfusion with ryanodine, repeated stimulations no longer elicit the Ca\(^{2+}\)/H11001 flash. C, Ca\(^{2+}\)/H11001 flash and wave probability from experiments as in panel B. The probability of evoking Ca\(^{2+}\)/H11001 flashes is not significantly affected by application of atropine but is almost abolished following exposure to ryanodine. Conversely, Ca\(^{2+}\)/H11001 waves are not observed in the presence of atropine. Note the higher probability of Ca\(^{2+}\)/H11001 flashes relative to Ca\(^{2+}\)/H11001 waves in control stimulations. D, Ca\(^{2+}\)/H11001 flashes occur through CICR. In the presence of 10 \(\mu\)M ryanodine (left) or 10 \(\mu\)M nitrendipine (right), the probability of observing postsynaptic Ca\(^{2+}\)/H11001 flashes is markedly reduced, whereas an increase in the average number of evoked Ca\(^{2+}\)/H11001 waves was observed (not significant). Experiment number indicated by parentheses. * and ** indicate significance at the 0.05 and 0.01 levels, respectively.
FIG. 6. Relationship between modes of postsynaptic Ca\(^{2+}\) signaling. A, individual myocytes are capable of both postsynaptic responses. Left, initial fluorescence of myocytes with the responding cell outlined. Right, pseudocolor images (cropped and rotated for clarity) show an initial Ca\(^{2+}\) flash followed by a Ca\(^{2+}\) wave in the same myocyte. The image at which the stimulus was applied is shown at left and indicated as time 0. Below, the average fluorescence values from the pixels identified by the white circle in the stimulus image are plotted, and the pseudolinescan from the pixels indicated by the dotted line in the stimulus image is shown. The pseudolinescan indicates the lower amplitude, simultaneous rise in
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aptic Ca$$^{2+}$$ signals within the same tissue segment are shown in Fig. 4; pseudolinescans constructed from a line of pixels drawn along the length of myocytes demonstrate slow Ca$$^{2+}$$ waves proceeding from one end of a cell to the other at a propagation rate of roughly 15 μm/s, whereas in Ca$$^{2+}$$ flashes wave propagation was not detected in x-y image sets acquired at 14 Hz.

Pharmacologic Dissection of Postsynaptic Ca$$^{2+}$$ Responses—We reasoned that the two distinct modes of postsynaptic Ca$$^{2+}$$ signaling likely reflect distinct neurotransmitter responses, which have been related to specific contractile responses in the detrusor (21). Previous reports in fluo-4-loaded smooth muscle tissues have identified nerve-evoked or spontaneous asynchronous, propagating Ca$$^{2+}$$ waves (22–25), and purinergic, postsynaptic subcellular junctional Ca$$^{2+}$$ transients (19, 24) in smooth muscle cells in situ. Asada et al. (20) have reported spontaneous Ca$$^{2+}$$ flashes in arterial myocytes. Dual modes of nerve-evoked Ca$$^{2+}$$ signaling in the same myocytes have not been reported in smooth muscle, however, and we therefore further investigated the nature of these Ca$$^{2+}$$ signals. Ca$$^{2+}$$ flashes occurred with a shorter lag time following stimulation, the kinetics of the rise and decay of the [Ca$$^{2+}$$], transient were markedly faster, and the peak [Ca$$^{2+}$$], obtained was significantly lower than observed in Ca$$^{2+}$$ waves (Fig. 5A). Nerve-evoked postsynaptic Ca$$^{2+}$$ flashes were resistant to atropine (1 μM; Fig. 5B) (although the frequency of events was decreased) and to xestospongin (a selective inhibitor of InsP$$^{3}$$ receptors) (1 μM, not shown), and the atropine-resistant Ca$$^{2+}$$ flashes were completely blocked by ryanodine (10 μM), indicating that they resulted from a rapid Ca$$^{2+}$$ release from ryanodine receptors (Fig. 5, B and C). Thus Ca$$^{2+}$$ flashes do not arise from the release of acetylcholine or the generation of InsP3. Conversely, Ca$$^{2+}$$ waves were never observed in the presence of atropine (1 μM; Fig. 5C) and were virtually abolished following the application of xestospongin (not shown), indicating that cholinergic signaling through InsP3, Ca$$^{2+}$$ release results in asynchronous Ca$$^{2+}$$ waves, consistent with previous reports (22, 24) that these signals arise from cholinergic signaling. Conversely, the purinergic receptor antagonist suramin blocked all Ca$$^{2+}$$ flashes, and all postsynaptic responses were also abolished by the combined action of atropine and the non-selective purinergic receptor antagonist suramin (data not shown). Selective desensitization of P2X1 receptors with α,β meATP (α,β meATP) further demonstrated that Ca$$^{2+}$$ flashes arise by stimulation of P2X receptors (see below). The rapid and synchronous nature of Ca$$^{2+}$$ flashes suggested that they arise through P2X-mediated depolarization and Ca$$^{2+}$$-induced Ca$$^{2+}$$ release, rather than simply by Ca$$^{2+}$$ influx through nonselective P2X channels. This hypothesis was confirmed in two ways. First, incubation with 10 μM ryanodine abolished virtually all Ca$$^{2+}$$ flashes without decreasing the probability of Ca$$^{2+}$$ waves (Fig. 5D), indicating that ryanodine receptor-mediated Ca$$^{2+}$$-release results in Ca$$^{2+}$$ flashes. Second, rapid inhibition of voltage-dependent Ca$$^{2+}$$ channels with nitrendipine (10 μM) also almost completely eliminated Ca$$^{2+}$$ flashes (Fig. 5D) without diminishing the probability of postsynaptic asynchronous Ca$$^{2+}$$ waves.

Relationship between Postsynaptic Ca$$^{2+}$$ Responses—Several aspects of the myocyte Ca$$^{2+}$$ signals were striking relative to the relationship between the two types of neurotransmitter responses. First, as stated, the probability of observing Ca$$^{2+}$$ flashes was markedly higher than that of Ca$$^{2+}$$ waves at low stimulation frequencies, which may result largely from prejunctional effects related to the stimulus-dependent release of ATP and acetylcholine (26) but may also relate to the intrinsic efficiency of the downstream coupling events. Second, we commonly observed Ca$$^{2+}$$ flashes followed by a Ca$$^{2+}$$ wave in the same cell (Fig. 6A), whereas Ca$$^{2+}$$ waves were never followed by Ca$$^{2+}$$ flashes during a single stimulation train, suggesting that Ca$$^{2+}$$ release by P2X/CICR occurred at a lower threshold of neural input and that muscarinic/InsP3 release depleted Ca$$^{2+}$$ stores sufficiently to render myocytes refractory to subsequent CICR. Third, low frequency stimulation commonly evoked repeated Ca$$^{2+}$$ flashes, whereas higher frequency stimulation resulted in a wave in the same cell (Fig. 6B), further indicating a higher threshold for muscarinic postsynaptic Ca$$^{2+}$$ responses.

The role of P2X receptors in rapid Ca$$^{2+}$$ flashes and the relationship between postsynaptic signaling modes in individual cells was established by using the selective P2X agonist α,β meATP, which rapidly desensitizes P2X1 receptors, highly expressed in urinary bladder smooth muscle (27, 28). In the presence of α,β meATP, Ca$$^{2+}$$ flashes were almost completely abolished and all postsynaptic signaling shifted to prominent, asynchronous Ca$$^{2+}$$ waves, the probability of which was markedly enhanced by P2X receptor desensitization (Fig. 6, C and D). The augmented probability of Ca$$^{2+}$$ waves at low stimulation frequency under conditions of P2X1 desensitization (and the increase in the probability of Ca$$^{2+}$$ waves in the presence of ryanodine or nitrendipine (Fig. 5D)) further suggest that the rapid release of Ca$$^{2+}$$ by ionotropic receptor stimulation and CICR raises the threshold for the slower activating and more sustained Ca$$^{2+}$$ release occurring through InsP3, R gating. Thus, these data suggest a degree of postsynaptic signal integration within myocytes.

**DISCUSSION**

The combination of transgenic, molecular engineering, and imaging technologies provides remarkable opportunities to design and target the expression of molecules that can be used to report critical cellular events in vivo (29–32). smGC mice reported here provide a means to efficiently examine physiological, postsynaptic signaling in vascular and nonvascular smooth muscle, and additional lines of mice with lineage-restricted expression of G-CaMP are likely to provide an important experimental advantage for the study of multicellular physiological processes in mammals. Moreover, crosses of these and other signaling mice with gene-targeted mice will provide a powerful tool to evaluate mammalian gene function. G-CaMP is robustly expressed in smGC mice and can be used to examine vascular and nonvascular Ca$$^{2+}$$ signaling in situ.

We have used G-CaMP transgenic mice to investigate postsynaptic Ca$$^{2+}$$ responses in individual myocytes in intact muscle of the urinary bladder. Our results indicate that indi...
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Cellular Ca\textsuperscript{2+} responses appear to increase the threshold for the metabotropic localized Ca\textsuperscript{2+} though the CICR process likely initiates with one or more as well as the poor quantum efficiency and slow transition rapidly purinergic Ca\textsuperscript{2+} remarkably distinct at the level of intracellular Ca\textsuperscript{2+} ionotropic (P2X) and metabotropic (muscarinic) receptors are arising from the separate actions of ATP and acetylcholine on postsynaptic responses, which are functionally linked at the probability of Ca\textsuperscript{2+} by ATP binding to P2X receptors and slower, InsP\textsubscript{3}-mediated release. That is, by releasing Ca\textsuperscript{2+} and graded, transient contractions, whereas increases in other interactions, however, such as the participation of ryanodine receptors. These data are consistent with numerous previous studies identifying cholinergic and purinergic excitatory neurotransmission and a prominent P2X component in visceral smooth muscles (27, 28, 33–35), extending information about these processes to the postsynaptic Ca\textsuperscript{2+} responses within individual myocytes in intact tissues. Evoked Ca\textsuperscript{2+} responses arising from the separate actions of ATP and acetylcholine on ionotropic (P2X) and metabotropic (muscarinic) receptors are particularly distinct at the level of intracellular Ca\textsuperscript{2+} release; rapid purinergic Ca\textsuperscript{2+} flashes result from CICR, mediated by P2X and channel gating and ryanodine receptor gating. Although the CICR process likely initiates with one or more localized Ca\textsuperscript{2+} sparks (36), the imaging bandwidth employed, as well as the poor quantum efficiency and slow transition kinetics of G-CaMP at low Ca\textsuperscript{2+} concentration (1), precluded identification of such events in these experiments. Measurements of Ca\textsuperscript{2+} signals in single myocytes correspond extremely well with a previous study of the neural basis of contractile responses within in individual myocytes in intact tissues. Evoked Ca\textsuperscript{2+} release in single myocytes strongly correlate with force production in the intact muscle.

Our findings also highlight the specific relationship between the different modes of postsynaptic Ca\textsuperscript{2+} signaling in smooth muscle. The rapid ionotropic response occurs with a distinctly lower stimulus threshold than metabotropic Ca\textsuperscript{2+} signaling, and the occurrence of Ca\textsuperscript{2+} release through an ionotropic response appears to increase the threshold for the metabotropic response, indicating an interdependence at the level of intracellular Ca\textsuperscript{2+} release. That is, by releasing Ca\textsuperscript{2+} from the sarcoplasmic reticulum, rapid Ca\textsuperscript{2+} flashes appear to decrease the probability of Ca\textsuperscript{2+} waves, thereby modulating the kinetics and intensity of muscle contraction. Our data do not exclude other interactions, however, such as the participation of ryanodine receptor Ca\textsuperscript{2+} release channels in the propagation of Ca\textsuperscript{2+} waves. Importantly, individual myocytes are capable of both postsynaptic responses, which are functionally linked at the level of Ca\textsuperscript{2+} release. These findings suggest a general mechanism by which a low frequency of firing of autonomic motor nerves results in a rapid mobilization of intracellular Ca\textsuperscript{2+} and graded, transient contractions, whereas increases in motor nerve depolarizations, through presynaptic mechanisms that result in augmented release of acetylcholine and shifts in the mode of postsynaptic signaling, result in slower Ca\textsuperscript{2+} waves and sustained contractions. This mechanism likely facilitates the rapid activation of detrusor muscle.

In summary, we report the production and use of transgenic mice for the investigation of Ca\textsuperscript{2+} signaling in living tissues. Advances in the quantum yield and stability of protein-based signaling molecules, as well as the generalization of this approach to the detection of other intracellular molecules, will likely markedly extend the scope and physiological relevance of studies of cell function.

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