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Coexistence of Functional IP_3 and Ryanodine Receptors in Vagal Sensory Neurons and Their Activation by ATP

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Hoesch, Robert E., Katherine Yienger, Daniel Weinreich, and Joseph P.Y. Kao. Coexistence of functional IP_3 and ryanodine receptors in vagal sensory neurons and their activation by ATP. *J Neurophysiol* 88: 1212–1219, 2002; 10.1152/jn.00155.2002. Intracellular photorelease of caged D-*myo*-inositol 1,4,5-trisphosphate (IP_3), caffeine application, and immunofluorescence confocal microscopy were used to determine that D-*myo*-inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) coexist in rabbit vagal sensory nodose ganglion neurons (NGNs). ATP, an extracellular physiological signaling molecule, consistently evoked robust transient increases in cytosolic free Ca^{2+} concentration (Ca^{2+} transients). ATP applied in Ca^{2+}-free physiological saline elicited Ca^{2+} transients that averaged approximately 70% of the amplitude of transients evoked in the presence of extracellular Ca^{2+}. The component of the ATP-evoked Ca^{2+} transient that was independent of extracellular Ca^{2+} corresponds to Ca^{2+} release from intracellular stores. This release component was sensitive to the pharmacological antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), U73122, neomycin, and heparin (13.5–15 kD), indicating that P2 purinoreceptors (P2Y) and the IP_3 signaling pathway are required for ATP-evoked Ca^{2+} release. Additionally, a portion of ATP-evoked Ca^{2+} release was inhibited by ryanodine, a selective blocker of RyRs. The ryanodine-insensitive component (approximately 70%) of ATP-evoked Ca^{2+} release corresponds to IP_3-induced Ca^{2+} release via IP_3Rs, while the ryanodine-sensitive component (approximately 30%) corresponds to consequent Ca^{2+}-induced Ca^{2+} release (CICR) via RyRs. These results indicate that functional IP_3Rs and RyRs coexist in nodose neurons and that both IP_3-induced Ca^{2+} release and CICR can be activated by ATP.

INTRODUCTION

Cytosolic Ca^{2+} ions are ubiquitous second messengers that regulate a broad spectrum of cellular processes, including membrane excitability (Hille 2001), ion channel gating (Hille 2001), gene expression (Bito et al. 1996), neurotransmitter release (Katz and Miledi 1968), muscle contraction (Fabiato and Fabiato 1975), and secretion of hormones (Curry et al. 1968) and digestive juices (Petersen 1992). Increases in cytosolic free Ca^{2+} ion concentration ($[Ca^{2+}]_i$) can result from Ca^{2+} influx through the plasma membrane, or Ca^{2+} release from intracellular Ca^{2+} stores through intracellular Ca^{2+} release channels.

Two types of intracellular Ca^{2+} release channels are known: ryanodine receptor (RyR) channels, and D-*myo*-inositol 1,4,5-trisphosphate receptor (IP_3R) channels (Hille 2001). While these channels both mediate Ca^{2+} release from intracellular stores, they differ in their mechanisms of activation. Ca^{2+} release via RyRs is activated by increases in $[Ca^{2+}]_i$ [Ca^{2+}-induced Ca^{2+} release (CICR)]. Typically, Ca^{2+} ions that trigger CICR arise from Ca^{2+} influx through plasma membrane voltage- or ligand-gated channels. In contrast, Ca^{2+} release through IP_3Rs is activated by increases in $[IP_3]$ (IP_3-induced Ca^{2+} release). IP_3 is normally generated through cleavage of phosphoinositide lipids by phospholipase C (PLC) coupled to cell-surface receptors (Berridge 1993).

CICR exists in many types of neurons, including sensory (Cohen et al. 1997; Shmigol et al. 1995), autonomic (Kuba et al. 1983), and CNS neurons (Irving et al. 1992; Llano et al. 1994). We have previously demonstrated the importance of CICR in primary vagal sensory neurons [nodose ganglion neurons (NGNs); for review see Cordoba-Rodriguez et al. 1999]. In NGNs, action potentials trigger transient rises in $[Ca^{2+}]_i$ (Ca^{2+} transients), which are produced by Ca^{2+} influx through voltage-gated Ca^{2+} channels and the consequent activation of CICR (Cohen et al. 1997). In a population of NGNs, action potential–induced CICR activates a K^+ current that underlies a membrane hyperpolarization lasting for several seconds after the action potential [slow afterhyperpolarization (sAHP)]. The major function of the sAHP is to control spike frequency adaptation in these neurons (Weinreich and Wonderlin 1987). Thus, in this population of NGNs, CICR plays a critical role in regulating membrane excitability.

In the present study, we investigate IP_3-induced Ca^{2+} release in NGNs. Using intracellular photorelease of caged IP_3, we determine that functional IP_3Rs exist in NGNs. We also show that extracellularly-applied ATP evokes intracellular Ca^{2+} release through IP_3 signaling. Furthermore, we show that CICR is a component of ATP-evoked Ca^{2+} release.

METHODS

Cell dissociation

New Zealand White rabbits of either sex, weighing 1–2 kg, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and killed by pentobarbital sodium overdose (100 mg/kg), as approved by the

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Institutional Animal Care and Use Committee of the University of Maryland Biotechnology Institute. Dissociated NGNs were prepared as described previously (Leal-Cardoso et al. 1993) with the exception that sterile technique was used and the final neuronal pellet was resuspended in Leibovitz L-15 medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS). The resulting cell suspension was plated as 0.2 ml aliquots onto 25-mm glass coverslips (Fisher Scientific, Newark, DE) coated with poly-D-lysine (0.1 mg/ml; Sigma, St. Louis, MO). NGNs were incubated at 37°C for 24 h, maintained at room temperature to prevent neurite outgrowth, and used for experiments for ≤72 h.

$[Ca^{2+}]_i$ measurements and calibration

Loading cells with fura-2 indicator, single-cell microfluorimetry, and calibration of ratiometric data and calculation of $[Ca^{2+}]_i$ were performed as previously described (Cohen et al. 1997; Kao 1994).

Fura-2 requires excitation by ultraviolet (UV) light, which could cause adventitious photolysis of caged IP_3. Therefore fluo-3, which is excited by visible light, was used to monitor $[Ca^{2+}]_i$ in all photorelease experiments. When fluo-3 was used, cells were loaded with fluo-3/AM in the same manner as fura-2/AM. Single-cell microfluorimetry was performed as previously described (Cohen et al. 1997), except that excitation was at 490 nm and that the fluorescence emission was passed through a 530-nm band-pass filter before photometric quantitation. We report fluo-3 fluorescence data as changes in fluorescence (ΔF) relative to baseline. The fluo-3 fluorescence intensity record was first corrected by subtracting the background fluorescence intensity, measured after cell lysis with digitonin (20 μM). A continuous baseline was obtained by performing a polynomial fit to the segments of the fluo-3 trace recorded between experimental manipulations. This baseline trace was subtracted from the background-corrected record to yield the ΔF trace.

Unless otherwise stated, the following conventions apply: *1*) numerical results are reported as a mean ± SE; *2*) when multiple responses were elicited from a NGN, the response amplitude under a given experimental condition was normalized to the control response amplitude; and *3*) Student's *t*-test (two-tailed) was used to assess significant differences between calculated means ($P < 0.05$ was considered significant).

Immunofluorescence microscopy

SOLUTIONS AND ANTIBODIES. Phosphate buffered saline (PBS) consisted of the following (in mM): 145 NaCl, 10 Na phosphate, and 10 NaN_3, pH 7.2. FBS-azide-NaCl-Tris (FANT) solution (1% or 10%) consisted of the following: 1% or 10% (vol/vol) fetal bovine serum, 10 mM NaN_3, 0.5 M NaCl, and 20 mM Tris, pH 7.2 (at 4°C). *p*-Phenylenediamine (PPD)-glycerol consisted of the following: 100 mg PPD dissolved in 10 ml of 1 M Tris (pH 8.5) mixed with 90 ml glycerol. Affinity-purified goat anti-IP_3R antibodies (GT328, raised against purified rat cerebellar IP_3Rs) and sheep anti-RyR antibodies (sheep 7037227), raised against purified rabbit skeletal RyRs, were gifts from Dr. Allan Sharp (Johns Hopkins University, Baltimore, MD) and Dr. Kevin Campbell (University of Iowa, Iowa City, IA), respectively. Fluorescent secondary antibodies (fluorescein-labeled donkey-anti-goat and donkey-anti-sheep) were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

ANTIBODY LABELING. NGNs plated on poly-D-lysine-coated No. 1 glass coverslips were washed in PBS, fixed in 2% paraformaldehyde in PBS for 15 min, washed three times in PBS, quenched 50 min in 0.1 M glycine in PBS (pH 8), permeabilized for 5 min in 0.5% Triton in PBS, and given three 20-min incubations in 10% FANT, followed by three 10-min incubations in 1% FANT. The neurons were labeled with primary antibody (goat-anti-IP_3R or sheep anti-RyR) for 20 h and washed for 8 h in 1% FANT; the wash solution was exchanged at 20-min intervals. Subsequently, the NGNs were labeled with fluorescent secondary antibody for 20 h and washed for 8 h in 1% FANT; the wash solution was exchanged at 40-min intervals.

CONFOCAL IMAGING. Coverslips bearing labeled NGNs were mounted in PPD-glycerol and examined on a laser scanning confocal microscope fitted with a ×63 oil-immersion objective with a N.A. of 1.4 (Model LSM 410; Carl Zeiss, Inc., New York). Fluorophores were excited by 488-nm light from an argon ion laser, and fluorescence emission was acquired through a 515- to 565-nm band-pass filter. A single 1.09-μm optical section was taken through the thickest part of each NGN.

Physiological saline solutions

EXTRACELLULAR SOLUTION. Neurons were superfused with physiological saline solution (21–24°C) that contained the following (in mM): 120 NaCl, 3.0 KCl, 1.0 NaH_2PO_4, 25.0 $NaHCO_3$, 1.5 $MgCl_2$, 2.2 $CaCl_2$, and 10.0 dextrose, equilibrated with 95%O_2-5%CO_2 and pH adjusted to 7.2–7.4. For experiments where nominally Ca^{2+}-free medium was required, $CaCl_2$ was omitted.

INTRACELLULAR SOLUTION. Patch-pipette stock solutions contained the following (in mM): 152 KCH_3SO_3, 10.0 HEPES, 2.0 $MgCl_2$, 1.0 Na_3ATP, 1.0 Na_3GTP, and 1.0 KCl; pH adjusted with KOH to 7.2. KCH_3SO_3 was used to avoid excess intracellular Cl^-, which is known to inhibit G proteins (Lenz et al. 1997). Aliquots of stock pipette solution were stored frozen at 0°C. Each aliquot of pipette solution was thawed, stored on ice, and used for only 1 d. K_5Fluo-3 was added to the pipette solution to a final concentration of 50 μM; sufficient $CaCl_2$ was added to set free $[Ca^{2+}] = 100$ nM (taking the Ca^{2+} dissociation constant of fluo-3 under physiological conditions to be 400 nM; Minta et al. 1989). For photolysis experiments, 0.5 mM of the trisodium salt of D-*myo*-inositol 1,4,5-trisphosphate $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged IP_3) was included in the pipette solution, which was loaded only into the tip of the pipette. The shaft of the pipette was filled with pipette solution containing no caged IP_3. For experiments using fura-2, the pipette solution contained 50 μM K_5Fura-2 and sufficient $CaCl_2$ to set $[Ca^{2+}]_i = 100$ nM (taking the Ca^{2+} dissociation constant of fura-2 under physiological conditions to be 224 nM; Grynkiewicz et al. 1985).

Reagent delivery

EXTRACELLULAR REAGENT DELIVERY. A custom recording chamber with a narrow rectangular flow path provided 7 ml/min superfusion of NGNs on a No. 1 glass coverslip via a gravity flow system. The chamber was mounted on the stage of an inverted microscope (Diaphot; Nikon, Melville, NY) equipped with a ×40 phase-contrast oil-immersion objective (Fluor, N.A. 1.3; Nikon) to allow fluorescence measurements or direct visualization of NGNs. In experiments where drugs were applied in Ca^{2+}-free solution, nominally Ca^{2+}-free physiological saline was superfused for ≥10 s before and after drug application. Solution changes were complete in 14 s, as determined with fluorescent tracers.

INTRACELLULAR REAGENT DELIVERY. The whole cell configuration of the patch-clamp technique was used to deliver membrane-impermeant reagents (caged IP_3 and heparin). Patch pipettes (2–3 MΩ), fabricated from 1.5 mm OD borosilicate glass stock (World Precision Instruments, Sarasota, FL) on a Flaming-Brown P97 micropipette puller (Sutter Instruments, Novato, CA), were used with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). NGNs were first loaded with fluo-3/AM or fura-2/AM. After forming a gigaohm seal (>1.0 GΩ), the whole cell configuration was established, with neurons voltage clamped to −50 mV. Neurons were considered suitable for study if membrane input resistance was >150 MΩ, holding current was <200 pA, and resting $[Ca^{2+}]_i$ was ≤100 nM. Because heparin can activate RyRs (Bezprozvanny et al. 1993), when heparin was used intracellularly, Ry (10 μM) was also included in the intracellular solution.

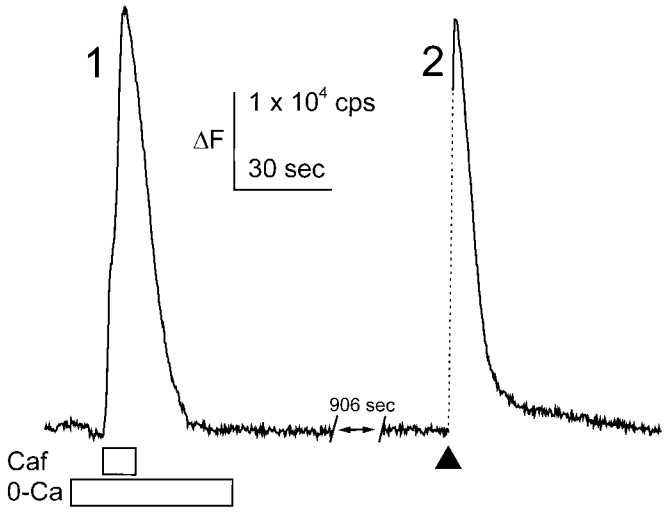

FIG. 1. Ca^{2+} transients evoked by caffeine and D-*myo*-inositol 1,4,5-trisphosphate (IP_3) photorelease recorded in the same nodose ganglion neurons (NGNs). *Trace 1*: Ca^{2+} transient evoked by a 10-s pulse of caffeine (Caf; 10 mM) in Ca^{2+}-free medium (0-Ca). Horizontal bars below the trace mark durations of reagent application. *Trace 2*: Ca^{2+} transient evoked by intracellular IP_3 photorelease effected by a 500-ms ultraviolet (UV) flash. The NGN was loaded with 500 μM caged IP_3 via whole cell patch pipette before recording trace. *1*: arrowhead marks the start of the UV laser pulse. The interval during which the UV flash precluded photometry is indicated by the dashed line. Fluo-3 was used to monitor changes in $[Ca^{2+}]_i$; results are presented as changes in fluo-3 fluorescence intensity (ΔF) in units of counts per second (cps).

FLASH PHOTOLYSIS OF CAGED IP_3. To photolyze caged IP_3, we delivered 500-ms flashes of UV light to NGNs loaded with caged IP_3. The multiline UV output (333.6–363.8 nm) of an argon ion laser (BeamLok 2065-7S; Spectra-Physics, Mountain View, CA) was used for photolysis. The output beam of the laser was directed through an objective lens (U-27X; Newport Corp., Irvine, CA) and focused onto the cleaved 50-μm diam silica core of a step-index multimode optical fiber (CeramOptec, East Longmeadow, MA). For adjusting alignment, the objective lens and the chuck holding the optical fiber were both mounted on a multimode fiber coupler assembly (F-915T; Newport Corp.). The output end of the optical fiber was sheathed in a glass pipette, which was mounted on a hydraulic micromanipulator to allow the output of the fiber to be directed onto cells being viewed under the microscope. The duration of UV flashes was regulated by a laser shutter (LS200F; NM Laser Products, Sunnyvale, CA) interposed between the laser head and the UV objective lens of the coupler. Shutter gating was controlled by TTL signals through pClamp8 software (Axon Instruments).

Reagents

Reagents were obtained from the following sources: caffeine from Sigma-Aldrich (Milwaukee, WI); ryanodine, caged IP_3, and heparin (13.5–15 kD) from Calbiochem (La Jolla, CA); acetoxymethyl esters of fura-2 (fura-2/AM) and fluo-3 (fluo-3/AM) and pentapotassium salts of fura-2 and fluo-3 from Teflabs, Inc. (Austin, TX); and neomycin, U73122, ATPγS, UTP, PPADS, and ATP from Alexis Biochemicals (San Diego, CA). Inorganic salts were from VWR (Piscataway, NJ).

Reagent solutions were prepared daily from concentrated stock solutions in dimethylsulfoxide (Fisher Biotech, Fair Lawn, NJ) or water that were stored frozen. Unless otherwise noted, drugs were delivered via the superfusate by switching a three-way valve to a reservoir containing a known concentration of the drug in the extracellular solution.

Reagent concentrations were as follows: ATP and ATPγS, 100 μM; UTP, 300 μM; caged IP_3, 500 μM; caffeine, 10 mM; ryanodine and PPADS, 10 μM; neomycin, 2 mM; U73122, 1 μM; and heparin (13.5–15 kD), 1 mg/ml.

RESULTS

Functional RyRs mediating robust CICR existed in all primary vagal sensory neurons (NGNs) examined (Cohen et al. 1997; Hoesch et al. 2001). In the current work, we ask whether IP_3Rs, another type of intracellular Ca^{2+} release channel, are co-expressed with RyRs in NGNs. To provide functional evidence for the co-expression of RyRs and IP_3Rs, we loaded NGNs with caged IP_3 via whole cell patch pipettes and used fluo-3 to monitor $[Ca^{2+}]_i$, as shown in Fig. 1. We first confirmed the presence of functional RyRs by applying caffeine, the classic pharmacological agonist of RyRs, in Ca^{2+}-free medium (Hoesch et al. 2001). Ca^{2+}-free solutions were used for caffeine applications because, in addition to activating RyRs, caffeine can activate Ca^{2+} influx in some NGNs (Hoesch et al. 2001). In Ca^{2+}-free medium, caffeine reliably elicited robust Ca^{2+} transients (Fig. 1, *trace 1*). The second Ca^{2+} transient in Fig. 1 (*trace 2*) was evoked by photolysis of caged IP_3 (IP_3 photorelease) in the same NGN, several minutes later, in Ca^{2+}-containing medium. Similar responses evoked by caffeine and IP_3 photorelease were observed in two other NGNs tested with this protocol. These results suggest that functional RyRs and IP_3Rs can coexist in the same NGN.

Immunofluorescence confocal microscopy also revealed that NGNs express both RyRs and IP_3Rs. Figure 2 shows optical sections through NGNs that were fixed, permeabilized, and

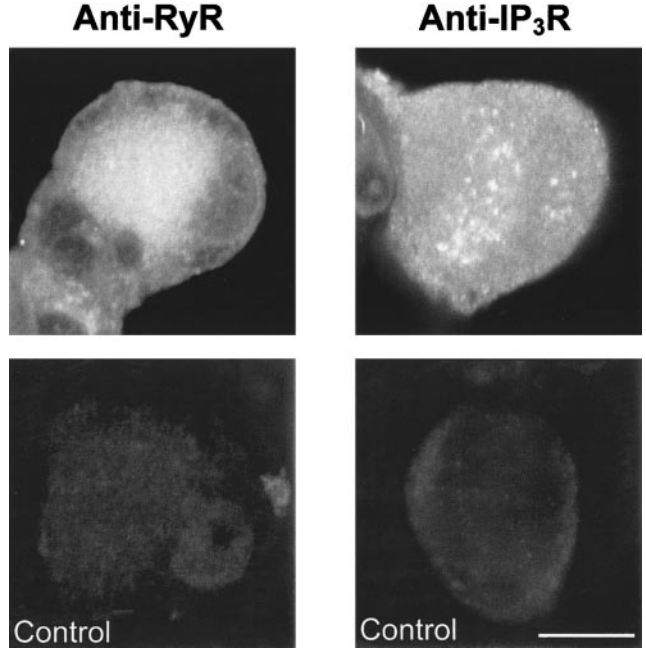

FIG. 2. Immunofluorescence labeling of ryanodine receptors (RyR) and IP_3 receptors. Laser scanning confocal images of NGNs showing immunofluorescence labeling with anti-ryanodine receptor antibody (*left column*, "anti-RyR") or anti-IP_3 receptor antibody (*right column*, "anti-IP_3R"). Fixed, permeabilized NGNs were first labeled with primary antibodies, followed by labeling with fluorescein-labeled secondary antibodies, and examined by laser scanning confocal microscopy. Control images (*bottom*) were obtained from NGNs that were treated identically, except that the respective primary antibodies were not included in the incubation solution during the 1st labeling period. Scale bar: 25 μm.

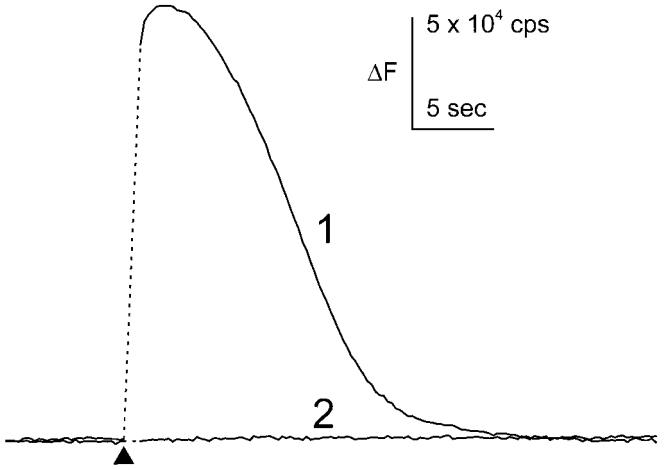

FIG. 3. Effect of heparin, an IP_3 receptor antagonist, on Ca^{2+} transients evoked by photoreleased IP_3. *Trace 1*: Ca^{2+} transient evoked by IP_3 photorelease effected by a 500-ms UV flash in a NGN loaded with 500 μM caged IP_3 via a whole cell patch pipette. *Trace 2*: in a different NGN, inclusion of heparin (13–15 kD, 1 mg/ml) in the intracellular pipette solution completely blocked the ability of photoreleased IP_3 to evoke a Ca^{2+} transient. In 3 NGNs, heparin completely inhibited Ca^{2+} transients evoked by IP_3 photorelease, indicating a role for IP_3Rs in generating these transients. Fluo-3 was used to monitor changes in $[Ca^{2+}]_i$; results are presented as ΔF in units of cps. Arrowhead marks the start of the UV laser pulses. The intervals during which the UV flash precluded photometry are indicated by the dashed lines.

labeled with anti-RyR or anti-IP_3R primary antibodies, and labeled with fluorescent secondary antibodies. These optical sections reveal both anti-RyR and anti-IP_3R staining, further supporting the conclusion that RyRs and IP_3Rs are both expressed in NGNs. In total, we imaged 29 anti-RyR-labeled NGNs and 36 anti-IP_3R-labeled NGNs. It is interesting to note that the subcellular distributions of RyR and IP_3R staining show some subtle differences. Anti-RyR staining occurs in two regions: a thin cortical band of staining, which is presumably sub-plasma membrane, and a larger region of staining in the interior of the cell. In contrast, anti-IP_3R staining appears to be more uniformly distributed throughout the cell. Moreover, bright punctate anti-IP_3R staining in the interior of the neurons was frequently observed, while anti-RyR staining tended to be more homogeneous in intensity. The significance of these apparent differences is not clear.

IP_3 photorelease consistently evoked Ca^{2+} transients in all NGNs tested (40 of 40). We sought to confirm that these Ca^{2+} transients evoked by IP_3 photorelease required functional IP_3Rs and resulted from release of Ca^{2+} from intracellular stores. When heparin, an IP_3R antagonist (Ehrlich et al. 1994), was included with caged IP_3 in the patch pipette, IP_3 photorelease did not evoke a detectable Ca^{2+} transient (Fig. 3, $n = 3$). Same-cell controls were not possible in heparin experiments because heparin was included in the pipette solution with caged IP_3, and thus was present throughout recording. Because heparin can activate RyRs (Bezprozvanny et al. 1993), Ry (10 μM) was also included in the pipette solution to block unintended RyR activation. Control experiments demonstrated that 10 μM Ry does not diminish the ability of photoreleased IP_3 to evoke Ca^{2+} transients ($n = 4$; data not shown). Given that IP_3 photorelease always evoked a Ca^{2+} transient in the absence of heparin ($n = 40$), the complete inhibition of the Ca^{2+} transient by heparin leads to three inferences. First, because heparin is an IP_3R antagonist, we infer that functional IP_3Rs are required for Ca^{2+} transients evoked by IP_3 photorelease. Second, two mechanisms could have potentially contributed to IP_3-evoked Ca^{2+} transients: Ca^{2+} release from intracellular stores and Ca^{2+} influx from extracellular medium. The finding that, in the presence of heparin, IP_3 photorelease caused no detectable change in $[Ca^{2+}]_i$ suggests that Ca^{2+} transients evoked by IP_3 photorelease are generated solely by intracellular Ca^{2+} release, with no contribution from Ca^{2+} influx. In separate experiments, we determined that amplitudes of Ca^{2+} transients evoked in the absence of extracellular Ca^{2+} averaged 1.03 $\pm$ 0.13 relative to same-cell control transients evoked in the presence of extracellular Ca^{2+} ($n = 6$; data traces not shown). Since Ca^{2+} transients evoked in the absence of extracellular Ca^{2+} are attributable to intracellular Ca^{2+} release, this finding further suggests that Ca^{2+} transients evoked by IP_3 photorelease are attributable to intracellular Ca^{2+} release alone. Third, since no change in $[Ca^{2+}]_i$ was detectable in the presence of heparin, we infer that UV light by itself does not activate Ca^{2+} release. This inference was further confirmed by delivering UV flashes to intact NGNs loaded with fluo-3, but no caged IP_3. In 10 NGNs tested, UV flashes alone never evoked Ca^{2+} transients (data not shown).

Next, we asked whether IP_3Rs can be activated with a physiological stimulus. In a variety of neurons, ATP is known to activate Ca^{2+} transients through IP_3-evoked Ca^{2+} release (reviewed by Dubyak and el-Moatassim 1993). However, determining the source of ATP-evoked Ca^{2+} transients can be complex when P2X (ionotropic) purinoreceptors are present in the plasma membrane. In rat NGNs, P2X activation causes influx of Na^+ and Ca^{2+} (Virginio et al. 1998), and a concomitant membrane depolarization, which can activate voltage-gated Ca^{2+} channels (VGCCs; Mendelowitz and Kunze 1992), permitting additional Ca^{2+} influx. Similar P2X-mediated effects were recorded in rabbit NGNs (unpublished observations). Therefore, to focus on ATP-activated intracellular Ca^{2+} signaling pathways, P2X-mediated Ca^{2+} signals were eliminated by excluding extracellular Ca^{2+}. Figure 4 shows typical ATP-evoked Ca^{2+} transients recorded with fura-2 indicator in

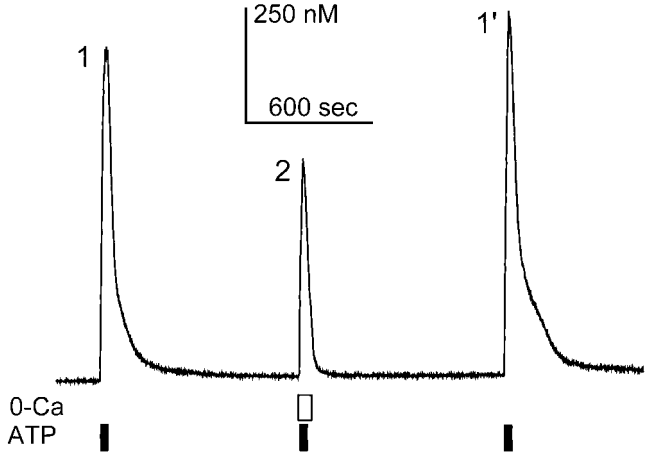

FIG. 4. Ca^{2+} transients evoked by ATP in the presence and nominal absence of extracellular Ca^{2+}. *1*: Ca^{2+} transient evoked by a 30-s pulse of ATP (100 μM) in normal physiological saline. *2*: Ca^{2+} transient evoked by ATP in the nominal absence of extracellular Ca^{2+} in the same NGN. *1′*: Ca^{2+} transient evoked by ATP after normal physiological saline was restored. These data indicate that intracellular Ca^{2+} release contributes significantly to ATP-evoked Ca^{2+} transients. Bars below the trace mark durations of reagent application. Fura-2 was used to measure $[Ca^{2+}]_i$.

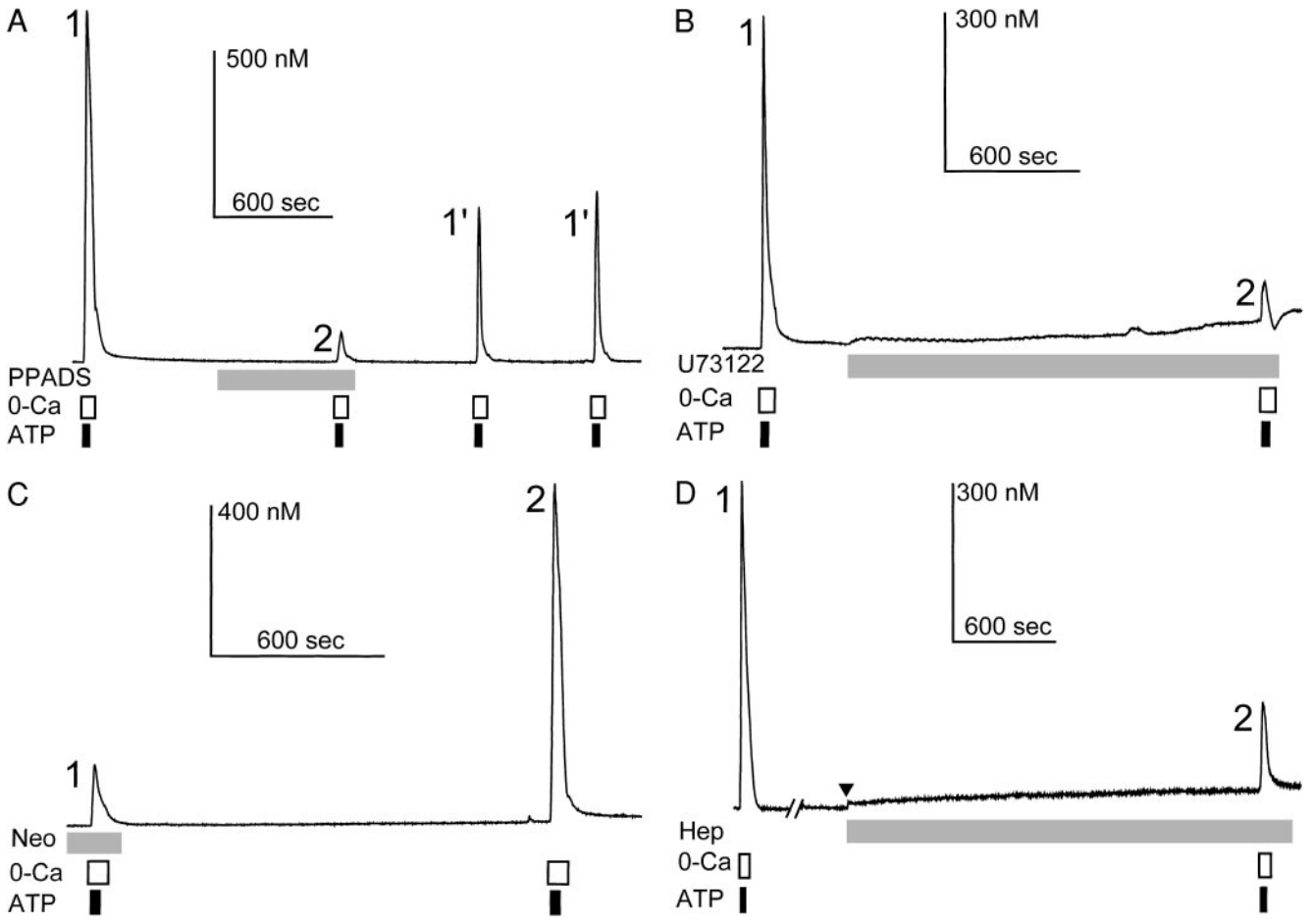

FIG. 5. Effects of various antagonists on ATP-evoked intracellular Ca^{2+} release. ATP (100 μM) was always applied in nominally Ca^{2+}-free physiological saline. *A*: 30-s pulse of ATP evoked intracellular Ca^{2+} release (*peak 1*). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS; 10 μM), an antagonist of P2 receptors, inhibited ATP-evoked Ca^{2+} release (*peak 2*). Washout of PPADS partially restored ATP-evoked Ca^{2+} release (*peaks 1′*). *B*: 30-s pulse of ATP evoked intracellular Ca^{2+} release (*peak 1*). U73122 (1 μM), an irreversible antagonist of phospholipase C, inhibited ATP-evoked Ca^{2+} release (*peak 2*). *C*: neomycin (Neo), an aminoglycoside that sequesters phosphoinositide lipids from cleavage by phospholipase C, reduced intracellular Ca^{2+} release evoked by a 30-s pulse of ATP (*peak 1*). To maximize sequestration of phosphoinositides before the start of measurement, NGNs were incubated with neomycin (2 mM) for ≥1 h. Washout of neomycin partially restored ATP-evoked Ca^{2+} release (*peak 2*). *D*: 30-s pulse of ATP evoked intracellular Ca^{2+} release (*peak 1*). Heparin (Hep; 1 mg/ml), an IP_3 receptor antagonist, inhibited ATP-evoked Ca^{2+} release (*peak 2*). Arrowhead marks the time when the whole cell configuration was established and intracellular delivery of heparin began. The break in the trace corresponds to the time interval (131 s) during which the patch pipette was positioned to form a gigaohm seal. Bars below the traces mark durations of reagent application. Fura-2 was used to measure $[Ca^{2+}]_i$.

the presence and absence of extracellular Ca^{2+}. In eight NGNs, the amplitudes of control pairs of ATP-evoked Ca^{2+} transients (Fig. 4, peaks 1 and 1′) averaged 576 ± 157 and 520 ± 112 nM, respectively, whereas those recorded in Ca^{2+}-free medium averaged 379 ± 91 nM. On average, 70.0 ± 5.0% of the amplitude of the ATP-evoked Ca^{2+} signal persists in the nominal absence of extracellular Ca^{2+} (Fig. 4, peak 2) and is therefore attributable to intracellular Ca^{2+} release. The component of the ATP-evoked Ca^{2+} transient that requires extracellular Ca^{2+} (approximately 30%) is attributable to Ca^{2+} influx, presumably via P2X channels and/or VGCCs (North and Barnard 1997).[1]

In other systems, ATP activates P2 purinoreceptors to trigger intracellular Ca^{2+} release (for review, see Dubyak and el-Moatassim 1993). To implicate P2Y receptors in ATP-evoked Ca^{2+} release in NGNs, we examined the effects of PPADS, a P2 purinoreceptor antagonist (Lambrecht et al. 1992), on ATP-evoked Ca^{2+} transients in Ca^{2+}-free medium. In the presence of PPADS, ATP-evoked Ca^{2+} release was significantly inhibited (Fig. 5*A*), averaging only 17.6 ± 3.5% of control values (Table 1). These results suggest that P2Y receptors are required for ATP-evoked Ca^{2+} release. To examine in more detail the involvement of P2Y receptors in Ca^{2+} release, we compared the efficacy of several nucleotides: ATPγS, ATP, and UTP. For these studies, we measured peak

[1] P2X-mediated Na^+ influx could conceivably influence ATP-evoked Ca^{2+} signaling by changing the driving force for Na^+/Ca^{2+} exchange (Blaustein and Lederer 1999). If Na^+/Ca^{2+} exchange is a significant Ca^{2+} extrusion mechanism in NGNs, then the decay of Ca^{2+} transients should be measurably faster in Ca^{2+}-free medium. For Ca^{2+} transients evoked by IP_3 photorelease and by ATP, the ratio of Ca^{2+} transient decay half-time in 0 Ca^{2+} to that in full Ca^{2+} in the same NGN averaged 0.95 ± 0.09 (n = 6) and 0.95 ± 0.17 (n = 8), respectively. Although Ca^{2+} transient decay seems to be slightly faster in Ca^{2+}-free medium (by approximately 5%), these ratios are not significantly different from 1. Therefore Na^+/Ca^{2+} exchange does not seem to be a significant Ca^{2+} extrusion mechanism in NGNs.

TABLE 1. *Effects of antagonists on ATP-evoked intracellular Ca^{2+} release**

Antagonist	Concentration	Site of Action	Percentage of Control	*n*
PPADS	10 μM	P2 receptors	18 ± 4.0	4
U73122	1 μM	PLC	5.0 ± 3.0	4
Neomycin	2 mM	Phosphoinositide lipids	49 ± 11	6
Heparin	1 mg/ml	IP_3Rs	37 ± 10	5
Ryanodine	10 μM	RyRs	71 ± 20	5

* ATP-evoked intracellular Ca^{2+} release was measured in the presence and absence of antagonist in Ca^{2+}-free medium, as illustrated in Figs. 5 and 6. The peak amplitude of each ATP-evoked Ca^{2+} transient in the presence of antagonist was normalized to a control transient in the same NGN. The normalized results were averaged and reported as the percentage of control ATP-evoked Ca^{2+} release remaining after antagonist treatment. Values are means ± SE.

agonist-evoked Ca^{2+} transients in the absence of extracellular Ca^{2+}, and normalized the peak value for a given agonist to the peak value for ATP in each NGN. Compared with ATP-evoked Ca^{2+} release, ATPγS-evoked Ca^{2+} release averaged 110 ± 30% for 7 NGNs, and UTP-evoked Ca^{2+} release averaged 16.0 ± 5.0% for 10 NGNs (responses not shown), indicating that ATP and ATPγS have similar efficacy for Ca^{2+} release and that both are significantly more effective than UTP. Such a rank-order of potency (ATPγS ≥ ATP ≫ UTP) does not match the published order for activation of any specific cloned P2Y receptor subtype (King et al. 1998), suggesting that more than one P2Y receptor subtype may be expressed in rabbit NGNs.

We probed the role of the IP_3 signaling pathway in ATP-evoked Ca^{2+} release by applying three antagonists: U73122, an inhibitor of PLC (Lee et al. 1998); neomycin (Neo), which complexes phosphoinositide lipids to render them unavailable as PLC substrates (Carney et al. 1985); and heparin (13.5–15 kD, Hep), an IP_3R antagonist (Ehrlich et al. 1994). Representative records showing inhibition of ATP-evoked Ca^{2+} release by each antagonist are shown in Fig. 5, (*B–D*), respectively. For each experiment, ATP-evoked Ca^{2+} release was measured in the presence and absence of antagonist. As shown in Fig. 5 (*B–D*), ATP-evoked Ca^{2+} release in the presence of antagonist was significantly reduced compared with control. The aggregate results from groups of NGNs treated as in Fig. 5 are summarized in Table 1. As shown in Table 1, each of the antagonists tested (U73122, Neo, and Hep) significantly inhibited ATP-evoked Ca^{2+} release, with inhibition ranging from 50 to 95%. Taken together, these data strongly suggest that ATP-evoked Ca^{2+} release is mediated by the IP_3 signaling pathway.

We note that none of the antagonists blocked ATP-evoked Ca^{2+} release completely. Inhibition of PLC by U73122 was most effective in blocking ATP-evoked Ca^{2+} release (95%). Sequestration of phosphoinositide substrates of PLC by neomycin appeared less effective (50%). Neomycin's inhibitory efficacy was likely underestimated in these experiments because same-cell control response was measured after neomycin washout. Washout of neomycin, a polycationic aminoglycoside, is expected to be inefficient and, thus may have been incomplete. Although heparin was quite effective in inhibiting ATP-induced Ca^{2+} release (63%), inhibition was nonetheless incomplete. In light of our finding that heparin *completely* inhibited the Ca^{2+} response evoked by IP_3 photorelease, the reduced effectiveness of heparin in blocking ATP-evoked Ca^{2+} release can be interpreted in at least two ways. First, in addition to IP_3R-mediated Ca^{2+} release, ATP signaling may engage another, IP_3R-independent, Ca^{2+}-mobilizing mechanism. This interpretation seems unlikely, however, because U73122 blockade of PLC inhibited ATP-evoked Ca^{2+} release almost completely (95 ± 3%), suggesting that IP_3 signaling is the major pathway engaged for ATP-evoked Ca^{2+} release. A second interpretation is that heparin may not have complete and uniform access to *all* subcellular spaces. The observation that heparin completely abolished Ca^{2+} transients evoked by IP_3 photorelease implies that heparin and caged IP_3 have equal access to the same spatial set of IP_3Rs. However, if there were additional subcellular spaces containing IP_3Rs inaccessible to heparin (and caged IP_3), but still accessible to activation by ATP, then ATP-evoked Ca^{2+} release would not be completely inhibited by heparin.

Knowing that ATP triggers intracellular Ca^{2+} release, we asked whether CICR through RyRs is a component of that release. If RyRs mediate such a component, then inhibition of RyRs with Ry should significantly attenuate ATP-evoked Ca^{2+} transients in Ca^{2+}-free medium. The trace in Fig. 6 is a representative record showing that Ry (10 μM) can significantly inhibit ATP-evoked Ca^{2+} release. Because RyR inhibition by Ry is time- and use-dependent (Meissner 1986; Sutko et al. 1985), Ry was first applied for ≥10 min, then caffeine (Caf) was repeatedly applied in the continued presence of Ry to facilitate RyR inhibition before a test pulse of ATP was applied. The amplitude of the ATP-evoked Ca^{2+} release in the presence of Ry was normalized to the amplitude of the control response in each NGN. The results from five NGNs revealed that, on average, 71 ± 20% of ATP-evoked Ca^{2+} release persisted in the presence of Ry (Table 1). This Ry-insensitive component is likely generated by release through IP_3Rs, while the Ry-sensitive component (approximately 30%) is attribut-

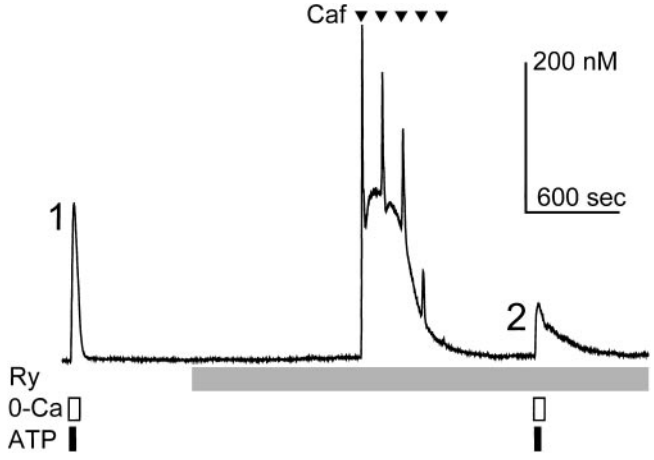

FIG. 6. Effect of ryanodine, an antagonist of Ca^{2+}-induced Ca^{2+} release, on ATP-evoked intracellular Ca^{2+} release. A 30-s pulse of ATP (100 μM) in nominally Ca^{2+}-free physiological saline evoked intracellular Ca^{2+} release (*peak 1*). Five 10-s pulses of caffeine (Caf; 10 mM, arrowheads) were applied in the presence of ryanodine (Ry, 10 μM) to ensure blockade of ryanodine receptors. Under persistent ryanodine receptor blockade, ATP-evoked intracellular Ca^{2+} release was significantly inhibited (*peak 2*). Slowing of the Ca^{2+} transient decay in the presence of Ry was a consistent observation. This may reflect concomitant inhibition of release-activated Ca^{2+} transport (RACT) when Ca^{2+}-induced Ca^{2+} release (CICR) was blocked by Ry (Cseresnyes et al. 1997). Time bars below the trace mark durations of reagent application. Fura-2 was used to measure $[Ca^{2+}]_i$.

able to release from RyRs (CICR). These results support the view that ATP activates IP_3-dependent Ca^{2+} release, which in turn, activates Ca^{2+}-induced Ca^{2+} release through RyRs.

DISCUSSION

Our major findings are as follows: *1*) all NGNs express functional IP_3 receptors; *2*) functional IP_3 receptors and ryanodine receptors can coexist within the same NGN; and *3*) ATP activates Ca^{2+} release through both IP_3 receptors and ryanodine receptors. A schematic diagram summarizing these results is presented in Fig. 7.

Previously, we observed that all NGNs exhibit robust CICR (Cohen et al. 1997; Hoesch et al. 2001), implying that all NGNs express functional RyRs. In the present study, intracellular photorelease of IP_3 triggered Ca^{2+} release in all 40 NGNs tested. This suggests that all rabbit NGNs also express functional IP_3 receptors. Indeed, application of caffeine, the classic RyR agonist, and IP_3 photorelease both evoked Ca^{2+} release in the same NGNs (Fig. 1). Immunofluorescence localization of antibodies to RyRs and IP_3Rs in NGNs further support these conclusions (Fig. 2). Together, these observations imply that functional IP_3Rs and RyRs coexist in the same NGNs.

The observation that intracellular Ca^{2+} release occurs through both IP_3Rs and RyRs in NGNs is significant for several reasons. First, that IP_3Rs and RyRs coexist in the soma of NGNs suggests that IP_3Rs and RyRs might also coexist within the central and peripheral processes of NGNs. Second, their different mechanisms of activation could allow both IP_3Rs and RyRs to be activated independently in response to complex stimuli such as peripheral inflammation. During inflammation, a variety of extracellular inflammatory mediators, such as 5-HT, bradykinin, and ATP, stimulate NGNs to fire action potentials (Undem and Carr 2001), which are known to activate CICR via RyRs (Cohen et al. 1997). Inflammatory mediators such as ATP (Dubyak and el-Moatassim 1993) could also simultaneously activate the IP_3 signaling pathway, leading to Ca^{2+} release via IP_3Rs. The consequent, amplified rise in $[Ca^{2+}]_i$ might then activate other cellular processes, including gene expression, an example of which could be the allergic

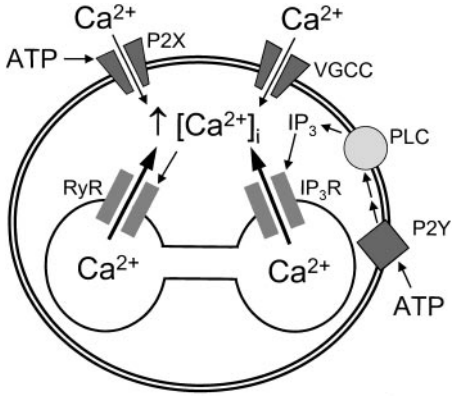

FIG. 7. Schematic diagram summarizing intracellular Ca^{2+} signaling mechanisms and their activation by P2 purinoreceptors in a NGN. P2Y activation by ATP stimulates IP_3 production by phospholipase C (PLC). IP_3 then triggers intracellular Ca^{2+} release through IP_3 receptors (IP_3Rs). The Ca^{2+} released through IP_3Rs can be further amplified by CICR through RyRs. Activation by ATP of ionotropic (P2X) purinoreceptors, and consequently, voltage-gated Ca^{2+} channels (VGCCs), can contribute Ca^{2+} influx.

inflammation-induced preprotachykinin gene expression in airway-projecting NGNs (Fischer et al. 1996). Third, since IP_3Rs and RyRs coexist, and their spatial distributions apparently overlap (Fig. 2), it is conceivable that Ca^{2+} could be released via IP_3Rs and RyRs from a common Ca^{2+} pool in NGNs, as has been shown in cerebellar Purkinje neurons (Khodakhah and Armstrong 1997). Alternatively, despite the overlapping spatial distributions of IP_3Rs and RyRs, the two channels may mediate Ca^{2+} release from distinct Ca^{2+} pools, as was observed in astrocytes and arterial myocytes (Golovina and Blaustein 1997, 2000). Distinguishing between these two alternatives requires further experimentation.

The existence of functional IP_3Rs in NGNs raises the question of the nature of the stimulus that might activate these receptors physiologically. ATP is one of many metabotropic agonists known to act through the IP_3 signaling pathway in other cells (Dubyak and el-Moatassim 1993). In the present work, ATP consistently evoked Ca^{2+} transients, which were predominantly (approximately 70%) attributable to intracellular Ca^{2+} release. In all NGNs tested, ATP could activate intracellular Ca^{2+} release that required metabotropic (P2Y) purinoreceptors and the IP_3 signaling pathway. However, P2Y receptors are unlikely to be the only receptors in NGNs that can signal through the IP_3 pathway. Metabotropic glutamate receptors (mGluRs), which are known to use the IP_3 signaling pathway in other cell types (Aramori and Nakanishi 1992), have been studied in (Hay and Kunze 1994) and recently cloned from rat NGNs (Hoang and Hay 2001). It is thus possible that the IP_3 signaling pathway in NGNs, in addition to being activated by ATP, may also be activated by glutamate, another physiological agonist.

NGNs are a heterogeneous collection of primary afferents that convey sensory information spanning a wide spectrum of modalities, including mechano-, thermo-, and chemo-sensation, from a broad range of visceral structures, including the small intestine, trachea, lungs, great vessels, and the stomach. In light of such diversity, the finding that all NGNs exhibit ATP-evoked Ca^{2+} transients that are mediated in part by IP_3Rs is of particular physiological interest. A role for ATP in nociceptive mechanosensory transduction in tubular (salivary duct, bile duct, vagina, and intestine) and saccular (urinary bladder, gall bladder, and lung) structures was recently hypothesized by Burnstock (2001). It was proposed that nerve fibers innervating the walls of such structures are sensitive to ATP released from distressed or damaged mucosal epithelial cells during mechanical stimulation (e.g., distension). Peripheral endings of NGNs also innervate tubular and saccular structures (intestine, blood vessels, stomach, trachea, and lungs) that are lined by epithelial cells. Therefore if NGN peripheral nerve endings, like the soma, are sensitive to ATP, then ATP released from damaged epithelial cells could stimulate NGN peripheral nerve endings by activating P2X and P2Y receptors. Activated P2X receptors conduct inward currents carried by Na^+ and Ca^{2+} ions (Thomas et al. 1998; Virginio et al. 1998), with the resulting membrane depolarization triggering Ca^{2+} influx through VGCCs (Mendelowitz and Kunze 1992). The P2X-mediated signals are paralleled by intracellular Ca^{2+} release though IP_3Rs triggered by P2Y receptor activation.

An important role of intracellular Ca^{2+} in NGNs is in the regulation of Ca^{2+}-activated K^+ channels (Cordoba-Rodriguez et al. 1999). We have previously shown in NGNs that CICR,

by activating K^+ channels, triggers a slow afterhyperpolarization (sAHP), which controls spike frequency adaptation (Moore et al. 1998; Weinreich and Wonderlin 1987). Our present study shows that ATP activates IP_3Rs and that Ca^{2+} released through IP_3Rs can activate CICR. Therefore ATP, as well as other metabotropic agonists, may control neuronal excitability through regulation of ion channels by IP_3-evoked Ca^{2+} release and/or consequent Ca^{2+}-induced Ca^{2+} release (see Fig. 7).

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