Purinergic Receptors Coupled to Intracellular Ca\(^{2+}\) Signals and Exocytosis in Rat Prostate Neuroendocrine Cells

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Rat prostate neuroendocrine cells (RPNECs) display a variety of ion channels and exhibit \(\alpha\)-adrenergic regulation of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{l}\)). In this study, purinergic regulation of [Ca\(^{2+}\)]\(_{l}\) and exocytosis was investigated in freshly isolated single RPNECs showing chromogranin A immunoreactivity. The presence of P2X and P2Y receptors in RPNECs was verified by the transient activation of Ca\(^{2+}\)-permeable cationic channels and the release of Ca\(^{2+}\) from intracellular stores by extracellular ATP, respectively. The transient inward cationic current was effectively activated by \(\alpha\beta\)-methylenedioxadenosine 5'-triphosphate (\(\alpha\beta\)-MeATP) and blocked by 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate, suggesting the presence of a P2X, or P2Y, subtype. For the release of stored Ca\(^{2+}\), ATP and UTP were equally potent, indicating the functional expression of the P2Y, or P2Y, subtype. The mRNAs for P2X, and P2Y, were confirmed from reverse transcription-PCR analysis of RPNECs. The application of \(\alpha\beta\)-MeATP induced large and transient increases in [Ca\(^{2+}\)]\(_{l}\), which were not attenuated by the blockers of voltage-activated Ca\(^{2+}\) channels or by depleting intracellular Ca\(^{2+}\) stores, but were abolished by omitting extracellular Ca\(^{2+}\). The application of UTP increased [Ca\(^{2+}\)]\(_{l}\) to 55% of the peak ∆[Ca\(^{2+}\)]\(_{l}\), induced by \(\alpha\beta\)-MeATP. The application of \(\alpha\beta\)-MeATP induced exocytotic responses of RPNECs as monitored by carbon fiber amperometry and capacitance measurements. To our interest, the application of UTP did not induce amperometric currents, but reduced the membrane capacitance, indicating a net endocytosis. From these results, we postulate that a sharp rise in [Ca\(^{2+}\)]\(_{l}\), by the P2X-mediated Ca\(^{2+}\) influx is required for exocytosis, whereas the relatively slow release of stored Ca\(^{2+}\) induces endocytosis in RPNECs.

Prostate neuroendocrine cells, considered to be a group of amine precursor uptake and decarboxylation cells, are intracellular regulatory cells with paracrine properties. Similar to other amine precursor uptake and decarboxylation cells in the gastric gland (1), prostate neuroendocrine cells may also influence the growth of the prostate gland or regulate the exocrine secretion of prostatic fluid (2, 3). Previous immunohistochemical studies suggest that prostate neuroendocrine cells both store and produce neurosecretory products such as serotonin, histamine, bombesin, calcitonin, and parathyroid hormone-related peptides that could regulate the growth, invasiveness, metastatic processes, and angiogenesis related to prostate carcinoma (4–7).

In a previous study, we initially reported that putative rat prostate neuroendocrine cells (RPNECs) display spontaneous action potentials and voltage-activated Ca\(^{2+}\) channels (both L and N types) in accordance with the electrically excitable properties of neuroendocrine cells (8). Also, pharmacological studies clearly demonstrated that RPNECs functionally express \(\alpha_1\) and \(\alpha_2\)-adrenergic receptors, which are linked to the release of Ca\(^{2+}\) and the inhibition of N-type Ca\(^{2+}\) channels, respectively (9). Despite the aforementioned electrophysiological evidence of excitable cells, the genuine property of neuroendocrine cells, viz. exocytosis, has not yet been directly investigated in RPNECs.

ATP is frequently colocalized with noradrenaline in postganglionic sympathetic nerve fibers and acts as a cotransmitter in several tissues (10). The purinoreceptors (P2), receptors for extracellular ATP, are divided into two distinct receptor families: G-protein-coupled metabotropic receptors (P2Y receptors) and receptor ion channels with a nonselective permeability to cations, including Ca\(^{2+}\) (P2X receptors) (10). In some neurons and neuroendocrine cells, extracellular ATP induces exocytosis in a Ca\(^{2+}\)-dependent manner. In the sympathetic neurons, for example, it has been suggested that the P2X receptors mediate a positive modulation of noradrenaline release, whereas the G-protein-coupled P2Y receptors mediate the opposite response (11). In the case of adrenal chromaffin cells displaying heterogeneous expression of P2X and P2Y receptors, P2X channels are preferentially localized to noradrenaline-secreting cells (12). The pathophysiological significance of purinoreceptors has also been suggested by the Ca\(^{2+}\) release and growth regulation of prostate tumor cells by extracellular ATP (13).

Considering the suspected role of ATP as a cotransmitter from the sympathetic nerves that regulate the function of prostate glands, we chose to investigate the operation of multiple Ca\(^{2+}\) translocation mechanisms linked to purinoreceptors in RPNECs. In addition, we compared the efficiency of the exocytosis triggered by P2X and P2Y receptors recruiting different sources of calcium ions in RPNECs. The direct measurement of

\[^{1}\text{The abbreviations used are: RPNECs, rat prostate neuroendocrine cells; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; [Ca}^{2+}]_l, cytosolic Ca}^{2+} \text{concentration; RT, reverse transcription; CFE, carbon fiber electrode; ATP}_{\text{S}}, adenosine 5'-O-(3-thiotriphosphate); \alpha\beta\text{-MeATP, } \alpha\beta\text{-methylenedinosine 5'-triphosphate; AMP-CPP, adenosine 5'-} (\alpha\beta\text{-methyl}-\text{enetriphosphate); TNP-ATP, } 2\text{'3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate; VOCCs, voltage-operated Ca}^{2+} \text{ channels.} \]
exocytosis in prostate neuroendocrine cells was performed for the first time in this study.

EXPERIMENTAL PROCEDURES

Cell Isolation—Male Sprague-Dawley rats (350–400 g) were killed by 100% CO2 inhalation, and the ventral prostate gland was removed rapidly thereafter. The procedure for single cell isolation was the same as described in our previous reports (8, 9). Briefly, the chopped tissue (1–2 mm3) was digested for 25 min at 37 °C in Ca2+-free phosphate-buffered saline (PBS) containing collagenase (2 mg/ml; Wako, Osaka, Japan), trypsin inhibitor (1 mg/ml; Sigma), bovine serum albumin (3 mg/ml; Sigma), and diithiothreitol (50 mg/ml; Sigma). Following digestion, tissue segments were transferred to fresh PBS and agitated gently using a fire-polished wide bore (1–2 mm) Pasteur pipette. Cells were isolated daily and stored in fresh solution at 4 °C for up to 6 h. Dispersed cells were moved into the experiment chamber and examined using an Olympus IX-70 inverted microscope. After the digestion procedure, most of the isolated single cells had an elongated columnar shape, a typical feature of secretory epithelial cells. In addition to columnar cells, we could identify round- or oval-shaped cells with a relatively dark cytoplasm that were regarded as RPNECs in this study.

Immunofluorescence Confocal Microscopy—Chromogranin A was identified using mouse monoclonal antibody LKH2H10 (NeoMarkers, Fremont, CA). Tissue segments were maintained at room temperature on a coverslip coated with polylysine (5%), fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and then incubated with PBS containing 3% bovine serum albumin (blocking solution) for 45 min. Cells were incubated with primary antibodies, followed by treatment with Alexa Fluor Fluor 546-coupled goat anti-mouse Ig (Molecular Probes, Inc., Eugene, OR) and double staining with fluorescein isothiocyanate (FITC)-conjugated phalloidin. Cells were then mounted onto glass slides and investigated using a Zeiss LSM 510 laser-scanning confocal microscope (FITC, excitation at 488 nm and emission at 520 nm; and Alexa Fluor 546, excitation at 556 nm and emission at 573 nm).

Cellular Ca2+ Measurement—Dispersed single cells were loaded with fura-2 acetoxyethyl ester (2 μM) in PBS for 20 min at room temperature and then washed out with fresh solution. RPNECs were identified following the above criteria, and the region of interest for the fura-2 experiment was set so that the fluorescence from a single RNPEC could be collected selectively. The recording of cytosolic Ca2+ concentration ([Ca2+]c) was performed with a microfluorometric system consisting of an Olympus IX-70 inverted fluorescence microscope with a dry-type fluorescence objective lens (×40, numerical aperture of 0.85), a photomultiplier tube (type R1527, Hamamatsu Photonics, Hamamatsu, Japan), and a Deltascan illuminator (Photon Technology International Inc., Lawrenceville, NJ). Light was provided by a 75-watt xenon lamp, and a chopper whose alternated the light beam into monochromators (340 and 380 nm) with a frequency of 16 Hz; the intensity of emitted light at 510 nm was meaasured. As a measure of [Ca2+]c, the fluorescence emission ratio at 340/380 nm excitation (F340/380) is presented.

Fluorescence Quenching Experiment—The divalent cation Mn2+ (0.1 mM) was used as a surrogate for Ca2+ to trace Ca2+ influx (14). Since fura-2 has a high affinity for Mn2+ (Kd = 5 mM), essentially all Mn2+ ions entering a fura-2-loaded cell are trapped as fura-2-Mn2+ complexes. These complexes are virtually non-fluorescent at all wavelengths. Thus, an accelerated attenuation of fura-2 fluorescence provides an estimate of the entry of divalent cations (e.g. Ca2+) into a cell.

Single Cell Reverse Transcription (RT)–PCR—To synthesize first strand cDNA, RPNECs were collected individually using microwelectodes with an inner diameter of ~25–30 μm on the stage of an inverted microscope for the patch clamp. After aspiration, the cell was expelled from the pipette into ice-cooled 0.2-ml tubes that contained 2 units of RNase-free DNase (Takara Bio Inc., Shiga, Japan), 40 units of ribonuclease inhibitor (Takara Bio Inc.), 50 mM Tris-Cl (pH 8.3), 3 mM MgCl2, 75 mM KC1, and 100 mM dithiothreitol. The reaction mixture was incubated at 37 °C for 30 min, followed by 95 °C for 5 min. The reverse transcription reaction was then performed in the presence of 0.8 μg of oligo(dT)12 (Roche Applied Science); 10 mM each dATP, dTTP, dGTP, and dCTP (Roche Applied Science); and 200 units of SuperScriptII™ reverse transcriptase (Invitrogen). The reaction proceeded for 10 min at 42 °C and followed by a 15-min step at 70 °C to inactivate the SuperScriptII™ reverse transcriptase.

Two rounds of PCR (PTC-0190 MiniCycler™, MJ Research, Inc., Waltham, MA) were performed with outer primers of P2Y2 (P2Y2F, P2Y2R), and P2X2 (P2X2F5, P2X2R), and P2X2 (15, 16) from rat and four sets of nested primers. First strand cDNA was used for the first PCR amplification. This mixture was then used for the second nested PCR amplification. PCRs were carried out using 2.5 units of Telzyme polymerase (Takara Bio Inc.) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 10 mM dATP, 10 mM dTTP, 10 mM dGTP, 10 mM dCTP, 100 μM each sense and antisense primer, and DNA template. Temperature cycling proceeded as follows: one cycle at 95 °C for 5 min and 45 cycles at 95 °C for 30 s, 50 °C for P2X2 or 55 °C for P2Y2, P2Y4, and P2X3, for 60 s, and 72 °C for 90 s, followed by 72 °C for 10 min. PCR products were then subjected to gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. The primers used for the PCR of P2Y2, P2Y2, P2X2, and P2X3 were as follows (15, 16): P2Y2 sense, 5′-CTG CCA GCC ACC CCT GTG CTA CTT-3′; P2Y2 antisense, 5′-CAT AGG TCA AGT GAT CAG GAG GAG-3′; P2Y2 nested sense, 5′-GTC ACC AGC AGC GTG ACG GGG-3′; P2Y2 nested antisense, 5′-GTA ATA GTA GGG GTG GCG GGG-3′; P2X2 sense, 5′-CAC CGA TAG CTG GTG ATC TGC CAC-3′; P2X2 antisense, 5′-CAG ACA GCA AAG ACA GTC ACC-3′; P2Y2 nested sense, 5′-CCT ACG GCA ATC CCT GGG-3′; P2X3 sense, 5′-GAA GCA TTC TAG GAG GGG-3′; P2X3 antisense, 5′-TTG ACA GTG GGC AGC TAG AT-3′; P2X3 nested sense, 5′-ATC ATC CCC ACC ATT ATC-3′; and P2X2 nested antisense, 5′-AAA TAG CAG CCC TTC TGC-3′.

Patch Clamp Methods—Isolated cells were transfected into a bath situated on the stage of an Olympus IX-70 inverted microscope. The bath (~0.3 ml) was superfused at 10 ml/min, and voltage clamp experiments were performed at room temperature (~22–25 °C). Patch pipettes (with a free tip resistance of ~2.5–3 megohms) were connected to the head stage of an Axopatch 1-D patch clamp amplifier (Axon Instruments, Inc., Foster City, CA). Liquid junction potentials were corrected with an offset circuit prior to each experiment. For the perforated whole cell patch clamp, a stock solution of nystatin in dimethyl sulfoxide (15 mg/ml) was added to the pipette solution, yielding a final concentration of 0.15 mg/ml. A steady-state perfusion (series resistance of <20 megohms) was usually achieved within 10 min after perfusion. A glass seal of pCLAMP 7.0 software (Axon Instruments, Inc.) was used for data acquisition and the application of command pulses. The voltage and current data were low pass-filtered (5 kHz) and displayed on a computer monitor. Current traces were stored in a Pentium-grade computer and analyzed using Origin Version. 6.1 (Microcal Software Inc., Northampton, MA).

Carbon Fiber Electrode (CFE) Amperometry—CFEs were fabricated from carbon fibers (5–11 μm diameter) and polypropylene micropipet tips of 10-μl volume as described previously (17). The tip of the electrode was closely apposed to the cell surface to minimize the diffusion distance from the release sites. The amperometric current, generated by the oxidation of released bioamines (e.g. serotonin) at the exposed tip of the CFE, was measured using an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) operated in the voltage clamp mode at a holding potential of 600 mV. Amperometric signals were low pass-filtered at 0.1 kHz with a gain of 20 or 50 mV/pA and then sampled at 0.5 Hz. Amperometric recordings were semiautomatically analyzed using software written in Igor (WaveMetrics, Lake Oswego, OR). Some recordings with a small number of amperometric signals were plotted on a fast chart recorder, and the events were counted manually. To evaluate the relative exocytosis, the numbers of exocytic events in the control and test conditions were averaged for 2 min, respectively.

Capacitance Measurements—Capacitance measurements were performed using the Lindau-Neher technique implemented as the “slopes” method (18) of the “software lock-in” mode of the extension of PClamp (HEKA Elektronik). A 1 kHz, 35-nA peak-to-peak sinusoid stimulus was applied about a direct current holding potential of ~60 mV. For the simultaneous acquisition of membrane current and capacitance, the X-chart program (HEKA Elektronik) was used.

Solutions and Chemicals—All experiments were performed in PBS containing 145 mM NaCl, 5.9 mM KCl, 0.8 mM KH2PO4, 0.4 mM KHCO3, 1 mM MgCl2, 2 mM CaCl2, and 5 mM v-glucose (pH 7.4) titrated with NaOH. CaCl2 was omitted in the enzymatic isolation of single prostate cells. The pipette solution for recording the P2X receptor current and resting membrane potential under nystatin-perfused conditions contained 100 mM CsCl, 1 mM MgCl2, 0.5 mM EGTA, 5 mM v-glucose, and 10 mM HEPES (pH 7.2) titrated with KOH. The pipette solution for the simultaneous measurement of membrane currents and [Ca2+]c, contained 130 mM CsCl, 1 mM MgCl2, 500 μM K+-fura-2, 5 μM v-glucose, and 10 mM HEPES (pH 7.2) titrated with CsOH. All drugs and chemicals were purchased from Sigma. The pipette solution for the
Identification of RPNECs and ATP-induced increase in \([\text{Ca}^{2+}]_i\). A, immunostaining of chromogranin A (panel a, red) and F-actin (panel b, green) in RPNECs. Isolated prostatic cells were stained with anti-chromogranin A mouse IgG and FITC-conjugated phalloidin. Visualization was realized with Alexa Fluor-coupled anti-mouse IgG and FITC. Panel c is a image of chromogranin A and F-actin. Bar = 10 \(\mu\text{m}\). B, representative trace of the fluorescence ratio \(F_{340/380}\) for ATP (100 \(\mu\text{M}\)) (black bars) in the presence and absence of extracellular \([\text{Ca}^{2+}]_o\) (Ca\(^{2+}\)-free, white bar). Note that the initial transient increase in \([\text{Ca}^{2+}]_i\), \(\Delta[\text{Ca}^{2+}]/\text{Ca}^{2+}\) transient by ATP (arrowhead) disappeared under the Ca\(^{2+}\)-free condition. The trace of the first response overlapped with that of the second response (red dotted line). The time break indicates 5 min. C, fluorescence intensities of fura-2 excited by wavelengths of 340 nm \((F_{340})\) and 380 nm \((F_{380})\). The \(y\) scale indicates the arbitrary unit (a.u.) of fluorescence. With MnCl\(_2\) (0.1 mM) in the Ca\(^{2+}\)-free bath solution (hatched bar), the application of ATP (100 \(\mu\text{M}\)) immediately attenuated both \(F_{340}\) and \(F_{380}\), followed by a symmetrical transient increase and decrease in \(F_{340}\) and \(F_{380}\) respectively.

Fig. 1. Identification of RPNECs and ATP-induced increase in \([\text{Ca}^{2+}]_i\). A, immunostaining of chromogranin A (panel a, red) and F-actin (panel b, green) in RPNECs. Isolated prostatic cells were stained with anti-chromogranin A mouse IgG and FITC-conjugated phalloidin. Visualization was realized with Alexa Fluor-coupled anti-mouse IgG and FITC. Panel c is an image of chromogranin A and F-actin. Bar = 10 \(\mu\text{m}\). B, representative trace of the fluorescence ratio \(F_{340/380}\) for ATP (100 \(\mu\text{M}\)) (black bars) in the presence and absence of extracellular \([\text{Ca}^{2+}]_o\) (Ca\(^{2+}\)-free, white bar). Note that the initial transient increase in \([\text{Ca}^{2+}]_i\), \(\Delta[\text{Ca}^{2+}]/\text{Ca}^{2+}\) transient by ATP (arrowhead) disappeared under the Ca\(^{2+}\)-free condition. The trace of the first response overlapped with that of the second response (red dotted line). The time break indicates 5 min. C, fluorescence intensities of fura-2 excited by wavelengths of 340 nm \((F_{340})\) and 380 nm \((F_{380})\). The \(y\) scale indicates the arbitrary unit (a.u.) of fluorescence. With MnCl\(_2\) (0.1 mM) in the Ca\(^{2+}\)-free bath solution (hatched bar), the application of ATP (100 \(\mu\text{M}\)) immediately attenuated both \(F_{340}\) and \(F_{380}\), followed by a symmetrical transient increase and decrease in \(F_{340}\) and \(F_{380}\) respectively.
ing by the Mn\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable channels activated by ATP, and the second symmetrical change, viz. the increase in \(\Delta F_{340/380}\), was regarded as the Ca\textsuperscript{2+} release from intracellular stores. These results suggest that RPNECs express both P2X and P2Y receptors, which recruit extra- and intracellular sources of Ca\textsuperscript{2+} with fast and slow kinetics, respectively.

P2Y-mediated Release of Ca\textsuperscript{2+} in RPNECs—UTP, a pyrimidine triphosphate, activates some subtypes of P2Y receptors and induces inositol 1,4,5-trisphosphate-mediated Ca\textsuperscript{2+} release (10). Thus, we compared the concentration dependence of stored Ca\textsuperscript{2+} release induced by extracellular ATP and UTP (Fig. 2, A and B). ATP or UTP was applied in the absence of extracellular Ca\textsuperscript{2+} to exclude the Ca\textsuperscript{2+} influx pathways. During the interval of ATP application, CaCl\textsubscript{2} (2 mM) was added back to replenish the intracellular calcium stores. The half-effective concentrations (EC\textsubscript{50}) of ATP and UTP were 2.8 and 3.8 \(\mu\text{M}\), respectively. The EC\textsubscript{50} of UTP in the presence of 2 mM CaCl\textsubscript{2} was 2.2 \(\mu\text{M}\), not significantly different from the EC\textsubscript{50} of UTP under the Ca\textsuperscript{2+}-free condition (Fig. 2B).

Next, the efficiency of stored Ca\textsuperscript{2+} release was compared between various analogs of purines and pyrimidines. As a positive control, a typical response of [Ca\textsuperscript{2+}]\textsubscript{c} to noradrenaline (10 \(\mu\text{M}\)) was confirmed for each cell (9). Then, the responses to ATP, UTP, UDP, 2-methylthio-ATP, ADP, and ATP\textsubscript{S}, all at 100 \(\mu\text{M}\), were compared in the absence of extracellular Ca\textsuperscript{2+}. The \(\Delta F_{340/380}\) were normalized to that by 10 \(\mu\text{M}\) noradrenaline in the control solution.

![Fig. 2. Subtypes of P2Y receptors coupled to the release of stored Ca\textsuperscript{2+}](image)

A, representative trace of \(\Delta F_{340/380}\) demonstrating the concentration-dependent effects of ATP (black bars) in the Ca\textsuperscript{2+}-free external solution (white bars). B, concentration-\(\Delta F_{340/380}\) relationships of ATP (\(n = 1\)) and UTP (\(n = 6\)) in the Ca\textsuperscript{2+}-free external solution and UTP in the control solution (\(n = 6\)). The peak amplitudes of \(\Delta F_{340/380}\) were normalized to the maximum effect by 100 \(\mu\text{M}\) ATP or UTP, and the mean values were fitted by a function (normalized \(\Delta F_{340/380} = 1/(1 + (\text{tested concentration/EC50}))\)). C, summary of \(\Delta F_{340/380}\) by various purinergic agonists under the Ca\textsuperscript{2+}-free condition. In each cell, \(\Delta F_{340/380}\) was normalized to that by 10 \(\mu\text{M}\) noradrenaline in the control solution. D, summary of the blocking effects of suramin (50 \(\mu\text{M}\)) on UTP-induced \(\Delta F_{340/380}\) (\(n = 7\)). E, single cell RT-PCR analysis of P2Y\textsubscript{2} and P2Y\textsubscript{4} receptors in RPNECs. 2-MeS-ATP, 2-methylthio-ATP.
P2Y2 compared with P2Y4 in rats (19). In RPNECs, the Ca2+/H11001
response to UTP (100 μM) was completely blocked by pretreat-
mint with 50 μM suramin (n = 7) (Fig. 2D). In addition, the
RT-PCR analysis of RPNECs consistently showed positive sig-
nals for P2Y2 (Fig. 2E).

Ca2+-permeable P2X Channels in RPNECs—Under the nys-
tatin-perforated or conventional whole cell patch clamp condi-
tions, extracellular ATP (10 μM) induced a fast transient in-
ward current (IATP) (Fig. 3A). In some cases, to ensure fast
solution exchange, 10 μM ATP was applied by puffing from a
thin polyethylene tube (tip diameter of −100 μm) located 50–
100 μm from the recorded cell. The “local puff” method, com-
bined with a nystatin-perforated whole cell clamp, was re-
quired to prevent the rundown of IATP following repetitive
stimulation (e.g. Fig. 3D).

The mean amplitude of IATP was −1.9 ± 0.78 nA at a holding
potential of −60 mV (n = 9). To obtain the I-V curve of IATP,
brief ramp pulses from 60 to −100 mV were applied, and the
control current was digitally subtracted from the peak current
in response to ATP. The I-V curve of IATP reversed direction at
10.0 ± 1.02 mV (n = 8) (Fig. 3B). In all cases, the IATP or
α,β-MeATP-induced inward current (see below) showed fast
inactivation, with 90% of the total current decayed within 1 s of
drug application.

The fast decay of IATP in RPNECs is a typical trait of P2X1
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Fig. 3. P2X receptors and ATP-induced inward current (IATP). A, original trace of IATP induced by a local puff of ATP (10 μM) under
nystatin-perforated patch conditions. B, I-V relationship of IATP obtained under conventional whole cell patch conditions. Ramp depolarization from
−100 to 60 mV (0.32 V/s, holding potential of −60 mV) was applied every second, and the I-V curve was obtained by the subtraction of the control
current from IATP. C, α,β-MeATP (10 μM)-induced transient inward current (holding potential if −60 mV) similar to IATP. D, inhibition of IATP by
TNP-ATP pretreatment. TNP-ATP (3 nM) was applied for 3 min (white bar) before the addition of ATP (black bars). The time break indicates 5 min.
E, RT-PCR analysis of P2X1 and P2X3 subtypes in single RPNECs. P2X1 signals were found in RPNECs. The signal for P2Y2 was confirmed as a
positive control. F, I-V relationship of the P2X receptor current obtained by ramp depolarization under bi-ionic conditions (pipette solution, 140 mM
Cs+; and bath solution, 110 mM Ca2+). G, concentration dependence of α,β-MeATP on [Ca2+]c with (■) or without (○) apyrase pretreatment (left
panel). Changes in F340/380 (ΔF340/380) induced by various concentrations of α,β-MeATP (right panel) are plotted and were fitted to obtain the EC50.
FIG. 4. Comparison of [Ca²⁺] responses induced by P2Y and P2X stimulations. A, no effect of α,β-MeATP (10 μM; black bars) on [Ca²⁺], in the absence of extracellular CaCl₂ (white bar). B, effects of noradrenaline (NA; 10 μM) and α,β-MeATP (10 μM; black bar) on [Ca²⁺], upon pretreatment with thapsigargin (TG; 1 μM; white bars) (left panel). Note that the second noradrenaline application had no effect, and the removal of extracellular Ca²⁺ (white bars) reversed the tonic increase in [Ca²⁺], by thapsigargin and noradrenaline. Summarized results are shown (right panel). C, representative response of [Ca²⁺], to α,β-MeATP (100 μM; black bars) and UTP (100 μM; black bar) (left panel). The second application of α,β-MeATP in the presence of nifedipine (Nif; 1 μM) and ω-conotoxin GVIA (ω-CTX; 1 μM; white bar) induced a large increase in
(AMP-CPP; 10 μM), a potent agonist of P2X₃ and P2X₇ receptors (20), induced a similar transient inward current with peak amplitude of 2.1 ± 0.22 nA at −60 mV (n = 22) (Fig. 3C). β,γ-MeATP (10 μM), another selective agonist of the P2X₃ and P2X₇ subtypes, also induced a similar inward current (n = 4) (data not shown). TNP-ATP (3 μM), a relatively selective antagonist of P2X₂ and P2X₃ (20), abolished 91 ± 2.4% (n = 4) of the Iₐ, in a reversible manner (Fig. 3D). Although the above pharmacological profiles do not discriminate between P2X₂ and P2X₃ subtypes, the single RT-PCR analysis indicated the presence of P2X₃ (but not P2X₇) transcripts in RPNECs (Fig. 3E).

P2X channels are usually more permeable to Ca²⁺ than monovalent cations and provide significant Ca²⁺ influx pathways in many kinds of cells (20–22). In RPNECs, after replacing the NaCl bath solution with 110 mM CaCl₂ solution, the bath application of 10 μM α,β-MeATP induced a huge inward current with a mean amplitude of 2.04 ± 0.41 nA (n = 7) at −60 mV. The I-V curve showed an inwardly rectifying property with a reversal potential of 18 ± 3.0 mV (n = 7) (Fig. 3F). Under this bi-ionic condition, where Ca²⁺ (140 mM) and Ca²⁺ (110 mM) are the only permeable cations at the intracellular and extracellular sides, respectively, the calculated permeability ratio of Ca²⁺ to Ca²⁺ (Pₐ/P₉ₐ) for the P2X channel is 2.0 (23). In line with the high Ca²⁺ permeability of P2X channels, the application of α,β-MeATP increased the Ca²⁺ concentration of RPNECs in a concentration-dependent manner (Fig. 3G). In some cases of this experiment, to prevent the spontaneous desensitization of P2X receptors by ATP released from nearby cells, apyrase (20 μg/ml) was included in the extracellular solution throughout the cell isolation and incubation period (22). The EC₅₀ of α,β-MeATP was 0.13 and 0.12 μM in the absence and presence of apyrase, respectively, indicating that the P2X channels were not significantly desensitized after single cell isolation (Fig. 3G).

In the following experiments, considering the fast desensitization, 10 μM α,β-MeATP was applied via the bath perfusate to ensure maximum and simultaneous stimulation of P2X receptors.

From the above results, it was clear that RPNECs express both P2X (P2X₃) and P2Y (P2Y₁₂) receptors that link to fast Ca²⁺ influx and relatively slow release of stored Ca²⁺, respectively. As a next step, we asked whether the P2X and P2Y receptors play a subtype-specific role in the physiological responses of RPNECs, viz. exocytosis. To address this question, we tested whether α,β-MeATP is a selective activator of P2X receptors without activating stored Ca²⁺ release. In the absence of extracellular Ca²⁺, α,β-MeATP had no effect on the [Ca²⁺]ᵢ of RPNECs at 10 μM (n = 7) (Fig. 4A) or 100 μM (n = 3) (data not shown). In another experiment, RPNECs were pretreated with noradrenaline (10 μM) in the presence of thapsigargin (1 μM), a potent inhibitor of Ca²⁺-ATPase in the endoplasmic reticulum. After confirming that noradrenaline did not release any additional Ca²⁺, α,β-MeATP (10 μM) was applied, which still induced a robust Ca²⁺ response (Fig. 4B). These results indicate that α,β-MeATP could be used as a selective agonist of P2X receptors mediating Ca²⁺ influx in RPNECs.

Despite the high Ca²⁺ permeability, the amount of direct Ca²⁺ influx via P2X₂ and P2X₇ channels is reportedly small because of their fast desensitization (22). According to the results obtained with heterologously expressed P2X channels (22), the Ca²⁺ influx via voltage-operated Ca²⁺ channels (VOCCs) secondarily activated by P2X₁- or P2X₇-induced depolarization greatly exceeds the Ca²⁺ influx via P2X₂/P2X₇ channels. Therefore, to block the L- and N-type VOCCs in RPNECs (8), cells were pretreated with nifedipine (1 μM) and ω-conotoxin GVIA (1 μM). However, the combined treatment with Ca²⁺ channel blockers did not affect the peak amplitudes of Ca²⁺ responses to α,β-MeATP (ΔF₄₀₀/₃₈₀ = 4.1 ± 0.45, n = 7) (Fig. 4C). In another experiment, the membrane voltage was clamped to −60 mV using a whole cell patch clamp to prevent the activation of VOCCs (Fig. 4D). Both the membrane current and the change in [Ca²⁺]ᵢ were simultaneously monitored using 0.2 mM Kₗ-fura-2 driedyze with a CaCl₂ pipette solution. Under these conditions, α,β-MeATP simultaneously induced a huge inward current and transient increase in [Ca²⁺]ᵢ (ΔF₄₀₀/₃₈₀ = 2.1 ± 0.37, n = 4). In the same cell, the increase in [Ca²⁺]ᵢ by a depolarizing step pulse to 0 mV was also measured. The mean of depolarization-induced ΔF₄₀₀/₃₈₀ was 0.8 ± 0.09 (n = 4), much smaller than that with α,β-MeATP (10 μM) (Fig. 4D). Thus, it is fairly safe to say that Ca²⁺ influx directly through α,β-MeATP-sensitive P2X channels determines the peak level of [Ca²⁺]ᵢ in RPNECs.

To compare the peak amplitudes of [Ca²⁺]ᵢ responses to P2X- and P2Y-selective stimulations, we applied α,β-MeATP (10 μM) and UTP (100 μM), alternating the order of drug application in each cell. Although both agents induced substantial increases in [Ca²⁺]ᵢ in all the putative RPNECs tested, the peak ΔF₄₀₀/₃₈₀ induced by α,β-MeATP was larger than that induced by UTP (ΔF₄₀₀/₃₈₀ = 3.8 ± 0.25 for α,β-MeATP and 2.1 ± 0.19 for UTP, n = 25; p < 0.05), as summarized in Fig. 5C. Apart from the larger peak amplitude, the recovery of the Ca²⁺ response after the washout of α,β-MeATP generally took longer time than that after the washout of UTP, the reason for which is not clear yet. In >90% of RPNECs tested for UTP and α,β-MeATP, both agonists induced significant increases in [Ca²⁺]ᵢ, indicating that P2X and P2Y receptors are coexpressed in the same cell.

Purinergic Stimulation and Exocytosis in RPNECs—Previous immunohistochemical studies suggest that prostatic neuroendocrine cells contain oxidizable amine compounds such as serotonin (5-hydroxytryptamine) (4, 24). Therefore, in an effort to prove exocytosis in putative RPNECs, we performed amperometry using a CFE (Fig. 5). The CFE was located in close contact with the cell, and the voltage was held at 600 mV. In 18 of 30 cells tested, the events of spontaneous exocytosis of oxidizable molecules were observed under the control conditions. The application of ATP (100 μM) increased the frequency and amplitudes of current spikes superimposed on a slowly fluctuating baseline current, as described previously for other cell types such as PC12 cells (25) and chromaffin cells (26). The time-expanded trace of an exocytotic event (Fig. 5D, inset) demonstrated an instantaneous upstroke in the oxidizing current, followed by an exponential decay. The burst-like increase of current spikes by ATP was in accordance with our hypothesis that an appropriate stimulation would induce the exocytosis of oxidizable compounds in putative RPNECs (Fig. 5A). The frequency of events rose from nearly 8.5/min in the control to 28/min after ATP application in this particular case (Fig. 5B). Both the amplitude and frequency of amperometric currents reached the highest levels at −10 s after the start of the application and then decreased to the basal levels despite the presence of ATP. Time-expanded traces of the control and stimulation with ATP are shown in Fig. 5C, where each spike had
an onset and exponential decay that are characteristic of exocytotic release from single secretory vesicles (27). To analyze the characteristics of quantal secretion in RPNECs more precisely, we measured the peak amplitudes and total charge of each exocytotic spike of the control and stimulation with ATP and then summarized the data in histograms (Fig. 5D). The average spike amplitude and area of quantal events were 0.24 ± 0.04 pA and 3.1 ± 0.41 femtocoulomb in the control and were significantly increased to 0.49 ± 0.07 pA and 9.3 ± 1.29 femtocoulomb by ATP treatment in this particular experiment. Similar results were obtained in the five RPNECs tested with ATP.

Stimulation of P2X (but Not P2Y) Induces Exocytosis in RPNECs—We then compared the effects of P2X and P2Y stimulations on the amperometrically measured exocytosis in RPNECs. α,β-MeATP (10 μM) significantly increased the amplitude and frequency of spikes in 14 of 15 cells tested. In
contrast, UTP (100 μM) induced a slight secretory response in only 2 of 15 cells. Fig. 6 depicts the representative results showing that only the stimulation with α,β-MeATP induced exocytotic responses; the frequency of spikes increased from 19 to 85 events/min (Fig. 6B). The difference between the two agonists is also obvious upon comparison of the histograms of the amplitudes and total charge of the current spikes (Fig. 6, C and D).

Although CFE amperometry allows a direct measurement of exocytosis, it is limited to detecting only oxidizable compounds. To overcome this limit, we adopted another method for measuring exocytosis, viz., the Lindau-Neher mode of capacitance measurement. The membrane capacitance ($C_m$), membrane current ($I_m$), and $[Ca^{2+}]_c$ of RPNECs were simultaneously monitored in the whole cell clamp mode. The representative cases show that application of α,β-MeATP (10 μM) induced a large inward current and $Ca^{2+}$ spike, followed by an increase in $C_m$ that decayed slowly to the control level (Fig. 7, A and B). In contrast, the $Ca^{2+}$ response to UTP (100 μM) was accompanied by a reversible decrease in $C_m$, implying endocytosis (Fig. 7A). In some cases of testing α,β-MeATP, the initial change in $C_m$ started from the level below the resting $C_m$ and then increased slowly to above the base line (trace not shown). Fig. 7B shows the mean time course of $C_m$ changes induced by α,β-MeATP and UTP in 23 and 15 RPNECs, respectively. On average, the maximum increase in $C_m$ by α,β-MeATP was 5.2 ± 1.53% of the control ($n = 23$), and the maximum decrease in $C_m$ by UTP was $-7.3 ± 1.39$% ($n = 15$).

Both the Δ$[Ca^{2+}]_c$ and exocytotic increase in $C_m$ by α,β-MeATP (10 μM) were abolished in the absence of extracellular $Ca^{2+}$ (1 mM EGTA without CaCl$_2$) despite the large inward current ($n = 6$) (Fig. 7, C and D). Similarly, the UTP-induced $C_m$ decrease was completely blocked when 10 mM EGTA was included in the pipette solution ($n = 5$) (data not shown), indicating that the endocytotic response to P2Y stimulation...
was also Ca\(^{2+}\)-dependent. Also, pretreatment of RPNECs with suramin (50 \(\mu\)M), but not with TNP-ATP (0.1 \(\mu\)M), blocked the endocytotic response to 100 \(\mu\)M UTP (n = 2) (Fig. 7E). Interestingly, upon pretreatment with TNP-ATP (0.1 \(\mu\)M), extracellular ATP consistently decreased the \(C_m\) of RPNECs (6.3 \(\pm\) 1.43%, n = 6) (Fig. 7E).

**DISCUSSION**

This study demonstrates that RPNECs express both P2X (P2X\(_1\)) and P2Y (P2Y\(_2\)) purinoreceptors. The fast Ca\(^{2+}\) influx via P2X channels seems to be more efficiently linked to exocytosis in RPNECs, whereas the relatively slow and smaller
increase in \( [Ca^{2+}] \) signaled by P2Y receptors is consistently linked to a net decrease in \( C_m \), viz. endocytosis. Previous studies of the effects of extracellular ATP in the prostate have been confined to epithelial cells (28) and smooth muscle (29). This study is, to our knowledge, the first investigation on the functional role of purinoreceptors in prostate neuroendocrine cells. Thus, like other organs, purinoreceptors seem to be widely distributed in the prostate gland, including neuroendocrine cells.

Both the fast desensitization of \( I_{ATP} \) and the potent activation by \( \alpha,\beta\)-MeATP are consistent with the known properties of P2X/P2X2 channels, and single cell RT-PCR confirmed the expression of the P2X2 subtype in RPNECs. It is noteworthy that the large \( \alpha,\beta\)-MeATP-induced increase in \( [Ca^{2+}]_c \) \((\Delta[Ca^{2+}]_c)\) was not significantly affected by blocking VOCCs or by depleting intracellular \( Ca^{2+} \) stores (Fig. 4). Because 10 or 100 \( \mu M \) \( \alpha,\beta\)-MeATP had no effect in the absence of extracellular \( Ca^{2+} \), the large \( Ca^{2+} \) response activated by \( \alpha,\beta\)-MeATP must have been due to the transmembrane influx of \( Ca^{2+} \). Considering the fast inactivation kinetics of P2X2 channels, however, it was surprising that the transient \( Ca^{2+} \) influx through P2X2 channels overwhelmed the contribution of VOCCs that would be concomitantly activated in RPNECs. In contrast to our present results, in GT1 cells heterologously expressing P2X1 or P2X2 receptors, the transient \( \Delta[Ca^{2+}]_c \), by extracellular ATP is largely abolished by nifedipine, suggesting that direct influx of \( Ca^{2+} \) via P2X2 and P2X1 plays a minor role (22). One plausible explanation of the unexpectedly strong influx of \( Ca^{2+} \) upon stimulation with \( \alpha,\beta\)-MeATP is the huge amplitude of the P2X receptor current in RPNECs. If we simply assume that \(-10\%\) of the 2 nA square current with a duration of 20 ms is carried by \( Ca^{2+} \), the transient \( Ca^{2+} \) entry is \(-4.145 \times 10^{-18} \) mol. Such flux of \( Ca^{2+} \) into a RPNEC with a diameter of \( 10 \) \( \mu M \) would produce an instantaneous increase in \( [Ca^{2+}]_c \), by 1.056 \( nm \). If only \( 1\% \) of the entering \( Ca^{2+} \) is present as a free ionized form, it will still produce \( 10 \mu M \Delta[Ca^{2+}]_c \), such a large \( \Delta[Ca^{2+}]_c \), might have not only overwhelmed the secondary influx of \( Ca^{2+} \) via VOCCs, but also induced \( \alpha,\beta\)-MeATP-dependent inactivation of VOCCs (23), which could explain the insignificant effects of \( Ca^{2+} \) channel blockers on \( \Delta[Ca^{2+}]_c \), induced by \( \alpha,\beta\)-MeATP (Fig. 4C).

The results of amperometry indicate that the tested cells are capable of synthesizing and releasing oxidizable bioamines (Fig. 5 and 6), most likely serotonin (4, 24). The single amperometric spikes in RPNECs usually display 5–10 ms of half-amplitude width. Such values are similar to those observed in pancreatic duct epithelial cells (7 ms) and adrenal chromaffin cells (5–15 ms) (17, 27). However, the mean amplitudes of these components determining the direction of responses and the threshold of \( \Delta[Ca^{2+}]_c \), will be essential to attain a full understanding of exo/endocytosis in RPNECs, a novel type of neuroendocrine cell.

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