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Potentiation of P2Y Receptors by Physiological Elevations of Extracellular K^+ via a Mechanism Independent of Ca^{2+} Influx

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ABSTRACT

Many physiological and pathophysiological situations generate a significant increase in extracellular K^+ concentration. This is known to influence a number of membrane conductances and exchangers, whereas direct effects of K^+ on the activation of G protein-coupled receptors have not been reported. We now show that Ca^{2+} release evoked by $P2Y_1$ receptors expressed in 1321-N1 astrocytoma cells is markedly potentiated by small increases in external K^+ concentration. This effect was blocked by the phospholipase-C inhibitor U-73122 (1-[6-[[17β]-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), but not by its analog U-73343 (1-[6-[[17β]-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione), and not by nifedipine, Ni^{2+}, Cd^{2+}, or Gd^{3+}. Thus, K^+ enhances D-myo-inositol 1,4,5-trisphosphate-dependent Ca^{2+} release without a requirement for Ca^{2+} influx. The cation dependence of this effect displayed the order $K^+ > Rb^+ >$ *N*-methyl-D-glucamine$^+$, and Cs^+ and choline$^+$ were ineffective. The potentiation by K^+ is half-maximal at an increase of 2.6 mM (total K^+ of 7.6 mM). K^+ caused a reduction in EC_{50} (2.7-fold for a 29 mM increase) without a change of slope; thus, the greatest effect was observed at near-threshold agonist levels. The response to K^+ can be explained in part by depolarization-dependent potentiation of $P2Y_1$ receptors [*J Physiol (Lond)* **555:**61–70, 2004]. However, electrophysiological recordings of 1321-N1 cells and megakaryocytes demonstrated that K^+ also amplifies ADP-evoked Ca^{2+} responses independently of changes in membrane potential. Elevated K^+ also amplified endogenous UTP-dependent Ca^{2+} responses in human embryonic kidney 293 cells, suggesting that other P2Y receptors are K^+-dependent. P2Y receptors display a widespread tissue distribution; therefore, their modulation by small changes in extracellular K^+ may represent a novel means of autocrine and paracrine regulation of cellular activity.

Virtually all cells generate a large outward concentration gradient for K^+, which is used to regulate the membrane potential and to transport ions or solutes. Although only small amounts of K^+ flow across the cell membrane during individual action potentials, it is well established that substantial increases in extracellular K^+ concentration ($[K^+]_o$) can occur over a sustained period of normal nerve or muscle activation, particularly where diffusion is limited by cellular architecture (Sykova, 1983; Sejersted and Sjøgaard, 2000). In addition, cellular damage or ischemia will generate substantial, larger increases in $[K^+]_o$ (Sykova, 1983). Various membrane proteins are known to be stimulated by an increase in external K^+, either directly as in Na^+,K^+-ATPase (Glynn et al., 1970), or as a result of K^+-induced membrane depolarization. Indeed, a large increase in external K^+ concentration is commonly used as a tool to induce membrane depolarization and to generate Ca^{2+} influx via voltage-gated Ca^{2+} channels in studies of excitable tissues. The activation of voltage-gated Ca^{2+} influx via K^+-dependent depolarization is also used physiologically in the adrenal glomerulosa cell as a mechanism of detecting small changes in plasma K^+ levels (Spat and Hunyady, 2004). This specialized response to K^+ results from a fine tuning of ionic conductances to allow voltage-gated Ca^{2+} influx, predominantly via T-type Ca^{2+} channels, to be stimulated by very small changes in membrane potential (Spat and Hunyady, 2004).

Seven transmembrane-spanning G protein-coupled receptors (GPCRs) are the largest family of surface proteins and

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ABBREVIATIONS: $[K^+]_o$, extracellular K^+ concentration; GPCR, G protein-coupled receptor; IP_3, D-myo-inositol 1,4,5-trisphosphate; NMDG, *N*-methyl-D-glucamine; DMEM, Dulbecco's modified Eagle's medium; AM, acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEK, human embryonic kidney; U-73122, 1-[6-[[17β]-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73343, 1-[6-[[17β]-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; MRS-2179, 2′-deoxy-N^6-methyladenosine-3′,5′-diphosphate; RT-PCR, reverse transcription-polymerase chain reaction.

are involved in the regulation of a wide range of physiological processes. Their activation mechanism is not normally considered to be directly regulated by $[K^+]_o$, although recent studies have suggested that a number of GPCRs may be sensitive to changes in the membrane potential (Martinez-Pinna et al., 2005). We now show that increases in extracellular K^+, including levels observed under physiological conditions (Sykova, 1983; Sejersted and Sjøgaard, 2000), markedly potentiate ligand-dependent activation of P2Y receptors. This response occurs in Ca^{2+}-free medium and in the presence of a variety of Ca^{2+} channel blockers, thus results from modulation of IP_3-dependent Ca^{2+} release without a requirement for Ca^{2+} influx. We also show that the underlying mechanism is in part independent of changes in membrane potential.

Materials and Methods

Solutions and Reagents. The standard external saline contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ 10 mM HEPES, 1 mM $CaCl_2$, and 10 mM D-glucose, pH 7.35 with NaOH. For Na^+-free saline, NaCl was replaced by an equal concentration of choline chloride. Elevation of K^+ or other cations was by equimolar substitution of the Cl^- salt for NaCl (or choline chloride), except for the experiment shown by the open column in Fig. 3, where K^+ was added without substitution. For Ca^{2+}-free saline, $CaCl_2$ was replaced by an equal concentration of $MgCl_2$. In patch-clamp experiments, the pipette saline contained 150 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES, 0.05 mM K_5fura-2, and 0.05 mM Na_2GTP (pH adjusted to 7.2 with KOH). Dulbecco's modified Eagle's medium (DMEM) and G418 (Geneticin) were from Invitrogen (Paisley, UK). K_5fura-2, fura-2AM, fluo-3AM, and fluo-4AM were from Molecular Probes (Leiden, The Netherlands). All other reagents were purchased from Sigma Chemical (Poole, Dorset, UK). ADP and 2-methylthio-ADP were treated by incubation with hexokinase and glucose, and ATP and 2-methylthio-ATP were treated with creatine phosphate/creatine phosphokinase, to remove contaminating triphosphate or diphosphate nucleotides, respectively, as described previously (Mahaut-Smith et al., 2000; Tung et al., 2004).

Cell Preparation. 1321-N1 astrocytoma cells, stably transfected (Tung et al., 2004) to express the human $P2Y_1$ receptor (1321-N1-*h*$P2Y_1$ cells), were grown in DMEM containing 10% fetal bovine serum; 1% penicillin, streptomycin, and amphotericin antibiotic antimycotic solution; and 600 μg/ml G418 at 37°C in a humidified atmosphere at 5% CO_2. Control experiments confirmed that the ADP-evoked Ca^{2+} responses in the cell clone used were caused by activation only of $P2Y_1$ receptors. First, the order of efficacy of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) responses was 2-methylthio-ADP > ADP > 2-methylthio-ATP > ATP, that being the known agonist profile of the $P2Y_1$ receptor (Nicholas et al., 1996; Leon et al., 1997). Second, the $P2Y_1$ receptor-specific antagonist MRS-2179 competitively inhibited the ADP-evoked Ca^{2+} response. Third, untransfected host cells gave no responses to ADP in the concentration range used in this study. HEK 293 cells were grown in DMEM supplemented with high glucose and L-glutamine and containing 10% fetal bovine serum and 1% penicillin, streptomycin, and amphotericin antibiotic antimycotic solution. Megakaryocytes from the femoral and tibial marrow of adult male Wistar rats were prepared for whole cell patch clamp as described in detail previously (Martinez-Pinna et al., 2005).

Intracellular Calcium Measurements in Cell Populations. Population measurements of $[Ca^{2+}]_i$ were made using a Flexstation II fluorimeter (Molecular Devices, Wokingham, UK). Cells were grown to a confluent monolayer in 96-well black-walled, clear-bottom Costar microtiter plates (Appleton Woods, Selly Oak, Birmingham, UK). Cells were loaded with fluo-4 by incubation with 2 μM fluo-4AM for 45 min at room temperature followed by a single wash. Excitation and emission wavelengths were 488 and 525 nm, respectively, and the emitted light was further filtered with a 515-nm long-pass filter. At the start of each experiment, the cells were bathed in either 200 or 150 μl of saline, for single and double addition experiments, respectively. Agonists, antagonists and high K^+ salines were added in 50-μl aliquots. For double addition experiments, the second addition always maintained the agonist/antagonist concentration achieved with the first addition.

Intracellular Calcium Measurements from Single Cells. $[Ca^{2+}]_i$ was measured at the single cell level using standard imaging or photometric techniques. 1321-N1-*h*$P2Y_1$ cells were grown on glass coverslips to ≥60% confluence. For imaging experiments, cells were loaded with fluo-3 by incubation with 2.5 μM fluo-3AM for 45 min at room temperature, followed by a single wash. In photometric experiments, fura-2 was included in the patch pipette, and ratiometric recordings were performed using a Cairn spectrophotometer system (Cairn Research Ltd., Kent, UK), during simultaneous whole-cell patch clamp, as described in detail previously (Martinez-Pinna et al., 2004). Fluorescence imaging was performed on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Welwyn Garden City, UK) with excitation at 488 nm and emission collected at >505 nm. The confocal pinhole was set to measure fluorescence from the entire cell thickness. Images were collected from fields of ~15 to 30 cells at a rate of 2 Hz.

Electrophysiology. Conventional whole cell patch-clamp recordings were conducted using an Axopatch 200 series patch-clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes had filled resistances of 3 to 3.5 MΩ. Megakaryocytes were held under voltage clamp, as described previously (Martinez-Pinna et al., 2005). Membrane potential was recorded from 1321-N1-*h*P2Y1 cells using the current-clamp (zero current) mode.

Reverse Transcription-Polymerase Chain Reaction. RT-PCR was used to detect mRNA for human $P2Y_2$ and $P2Y_4$ in HEK 293 cells. Total RNA was extracted using the RNeasy mini kit (QIAGEN, Dorking, Surrey, UK), and cDNA was prepared using the Omniscript RT kit (QIAGEN). Forward and reverse oligonucleotide primers were as described previously (Jin et al., 1998). After initial denaturation for 135 s at 95°C, 35 PCR cycles with 5 U/μl *Taq* polymerase (QIAGEN) were conducted as follows: denaturation at 95°C for 40 s, annealing at 65°C ($P2Y_4$) or 55°C ($P2Y_2$) for 40 s and extension at 72°C for 40 s, followed by 10 min at 72°C. Controls to verify that amplified products were not derived from genomic DNA omitted the reverse transcriptase during the RT step, but they were otherwise identical.

Data Manipulation and Statistics. Experiments shown for single cell recordings are representative of at least five other cells. Fluo-4 and fluo-3 fluorescence signals (*f*) were expressed as f/f_0 ratios to normalize to the fluorescence level at the start of the experiment (f_0). Background-corrected fura-2 values of 340/380-nm ratio were converted to $[Ca^{2+}]_i$ as described previously (Martinez-Pinna et al., 2005). All experiments were conducted at room temperature (22–25°C). Data were exported for analysis and fitting of concentration response relationships within OriginLab Origin version 6.0 (OriginLab Corp., Northampton, MA). Data are expressed as the means ± standard error of the mean, with statistical difference assessed using Student's unpaired *t* test. Statistical significance in the figures is shown at levels of $p < 0.05$ (*), 0.01 (**), or 0.005 (***).

Results

Potentiation of $P2Y_1$ Receptor-Evoked Ca^{2+} Release by Extracellular K^+. Application of 100 nM ADP to 1321-N1-*h*P2Y1 cells generated an initial transient (<50 s) increase in $[Ca^{2+}]_i$ followed by a constant $[Ca^{2+}]_i$ indistinguishable from that of the resting state. Figure 1A shows an example of the response at the single cell level measured by fluorescence imaging. An increase in $[K^+]_o$ of 30 mM with equimolar reduction in Na^+ (final K^+ and Na^+ concentrations of 35 and 115 mM, respectively) had no effect in the absence of agonist (Fig. 1B), demonstrating the lack of intrinsic K^+ dependence and thus also voltage-dependent Ca^{2+} influx or release under these conditions. However, the same increase in $[K^+]_o$ induced substantial $[Ca^{2+}]_i$ transients in the presence of ADP (Fig. 1B). This response was specifically caused by the increase in K^+, not the simultaneous decrease in Na^+, since no change in $[Ca^{2+}]_i$ was observed if 30 mM Na^+ was replaced by choline$^+$ (Fig. 3 and *Ability of Other Cations to Modulate the ADP-Evoked Ca^{2+} Response*). Potentiation of ADP-dependent Ca^{2+} responses by an increase of extracellular K^+ was still observed in Ca^{2+}-free medium (Fig. 1C), and thus results from release of internally stored Ca^{2+} rather than activation of latent Ca^{2+} channels or reversed $Na^+/K^+/Ca^{2+}$ exchange. Potentiation of $P2Y_1$ receptor Ca^{2+} responses by a 30 mM increase in $[K^+]_o$ was also observed in salines in which all Na^+ was replaced with choline$^+$ (Fig. 1D). This rules out an involvement of the Na^+,K^+-ATPase, for example via changes in internal Na^+, because the K^+ dependence of this pump is saturated at a $[K^+]_o$ of 5 mM under Na^+-free conditions (Glynn et al., 1970). It is noteworthy that enhancement of $P2Y_1$ responses was observed after smaller increases in $[K^+]_o$, even 1.5 mM (Fig. 1E), which is equivalent to the shift in $[K^+]_o$ that has been estimated to occur in skeletal muscle T-tubules under physiological conditions (Sejersted and Sjøgaard, 2000). A 1.5 mM increase in $[K^+]_o$ evoked a single $[Ca^{2+}]_i$ transient, whereas the response to a 30 mM increase was more robust, often causing multiple Ca^{2+} spikes (compare Fig. 1, B and E). However, because of significant heterogeneity in the magnitude of the Ca^{2+} response to 100 nM ADP, possibly resulting from variability in receptor density, the concentration dependence to the K^+ effect was not further examined at the single cell level. Nevertheless, it was of particular interest that K^+ could induce a $[Ca^{2+}]_i$ increase in some cells that failed to respond to the agonist alone (Fig. 1F). Overall, therefore, the data in Fig. 1 demonstrate that $P2Y_1$ receptor responses are markedly potentiated by small increases in $[K^+]_o$ within the concentration range that cells will experience under physiological and pathophysiological conditions (Sykova, 1983; Sejersted and Sjøgaard, 2000).

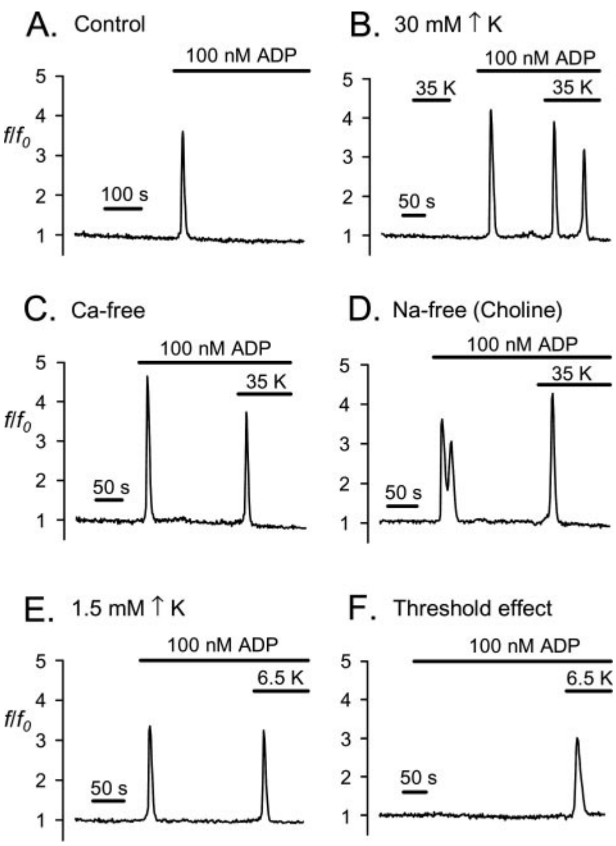

Fig. 1. Potentiation of $P2Y_1$ receptor-evoked intracellular Ca^{2+} responses by extracellular K^+ in single 1321-N1 cells. A to F show the intracellular Ca^{2+} response from a single 1321-N1-$P2Y_1$ cell representative of 15 to 30 cells within a semiconfluent layer studied by fluorescence imaging. The f/f_0 fluo-3 fluorescence ratio is used to indicate cytosolic Ca^{2+} levels. At the start of the experiments, cells were bathed in saline containing 5 mM K^+, with either 145 mM Na^+ (A–C, E, and F) or 145 mM choline$^+$ (D). The bars indicate addition of 100 nM ADP and elevation of external K^+ from 5 mM to either 35 mM (A–D) or 6.5 mM (E and F) with equimolar reduction of Na^+ (A–C, E, and F) or choline$^+$ (D). All salines contained 1 mM external Ca^{2+} except in C, which was nominally Ca^{2+}-free throughout. A to E show typical responses to increased saline K^+ concentration in cells that responded to ADP. F shows the typical response for a cell that failed to respond to ADP alone but then responded to increased K^+.

Extracellular K$^+$ Decreases the EC$_{50}$ for ADP at the P2Y$_1$ Receptor. To further characterize the effect of K^+ on $P2Y_1$ receptors, we measured average ADP-evoked $[Ca^{2+}]_i$ increases in 1321-N1 cells using a Flexstation II 96-well fluorimeter. The concentration-response curve for the ADP-stimulated peak $[Ca^{2+}]_i$ increase was shifted to the left by an increase in $[K^+]_o$, without a significant change in maximum response or slope ($p > 0.05$; Fig. 2A). The average EC_{50} for ADP was shifted 2.7-fold by a 29 mM increase in $[K^+]_o$ (53 ± 8 nM, $n = 6$, in 5 mM K^+; 20 ± 4 nM, $n = 6$, in 34 mM K^+; $p < 0.05$). Thus, as observed at the single cell level, the most dramatic enhancement of $P2Y_1$ responses by K^+ occurred at threshold concentrations of ADP (for example, 10 nM; Fig. 2B). Increased $[K^+]_o$ potentiated $P2Y_1$ receptors in a concentration-dependent manner (Fig. 2C), with half-maximal enhancement of the standard response in normal saline after an increase of 2.6 mM K^+ (total $[K^+]_o$ level of 7.6 mM). K^+ also caused a concentration-dependent potentiation of $P2Y_1$ receptors when increased from a starting level of zero, in which case a half-maximal effect was observed at 4.2 mM (not shown). For Fig. 2, A to C, ADP was premixed with high K^+ saline; however, K^+ also enhanced the average $P2Y_1$ response when increased after the initial agonist-evoked $[Ca^{2+}]_i$ increase (Fig. 2D, trace 1), as described above at the single cell level (Fig. 1). The lack of effect of saline addition in the presence of the agonist (Fig. 2D, trace 2), or of either saline addition or elevation of K^+ in the absence of agonist (Fig. 2D, trace 3), confirms that mechanical release of nucleotides (Lazarowski et al., 2000) did not contribute to the responses measured in this 96-well fluorimeter.

Ability of Other Cations to Modulate the ADP-Evoked Ca^{2+} Response. An increase in external divalent cation concentration (Mg^{2+} or Ca^{2+}) in the range 1 to 10 mM caused a concentration-dependent decrease in ADP-evoked Ca^{2+} responses (not shown) as reported previously for $P2Y_1$ receptors in platelets (Hall et al., 1994). However, other monovalent cations could substitute for K^+ in the potentiation of the ADP-evoked Ca^{2+} response in the 1321-N1-$hP2Y_1$ cell (Fig. 3). The ability to enhance the initial Ca^{2+} increase evoked by 100 nM ADP displayed the order of potency: $K^+ >$

$Rb^+ > NMDG^+$, whereas Cs^+ and choline$^+$ were ineffective when the concentration of each ion was increased by 30 mM with an equimolar decrease in Na^+. The lack of effect of Cs^+ and choline$^+$ increases suggest that a decrease in external Na^+ has little or no role in the response to K^+ or other monovalent cations. This was confirmed by the marked enhancement of ADP-mediated Ca^{2+} responses when K^+ was increased by 30 mM without substitution, whereas an additional 30 mM NaCl had no effect (Fig. 3, open columns).

The Potentiation of $P2Y_1$ Receptors by K^+ Does Not Require Activation of Voltage-Dependent Calcium Channels. In excitable cells, the main mechanism whereby an increase in extracellular K^+ can stimulate a $[Ca^{2+}]$ response is via membrane depolarization and activation of voltage-gated Ca^{2+} channels. Indeed, in adrenal glomerulosa cells increases in external K^+ of only 1 to 2 mM can generate substantial voltage-dependent Ca^{2+} influx (Spat and Hunyady, 2004). However, in the 1321-N1-*h*$P2Y_1$ cells, K^+ still potentiated the response to ADP in the presence of blockers of voltage-gated Ca^{2+} channels, including Ni^{2+} (200 μM), Cd^{2+} (100 μM), and nifedipine (10 μM) (Fig. 4). At 100 μM, La^{3+} and Gd^{3+} abolished the ADP-evoked responses in normal and 34 mM K^+ (not shown), suggesting that at high concentrations these common tools used to inhibit Ca^{2+} influx were directly interfering with activation of the $P2Y_1$ receptor. However, the enhancement of the response to ADP by elevated K^+ was maintained in the presence of 1 μM Gd^{3+} (Fig. 4B), a concentration of this multivalent cation reported to block store-dependent (capacitative) calcium entry (Broad et al., 1999). Together with the observation that the response is present in Ca^{2+}-free medium (Fig. 1C), these data demonstrate that K^+ can enhance ADP-dependent activation of $P2Y_1$ receptors via a mechanism independent of Ca^{2+} influx. The small reduction in $[Ca^{2+}]$ increase evoked by either ADP or ADP/K in the presence of Ni^{2+}, Cd^{2+}, and Gd^{3+} compared with the control, can be explained by the inhibitory effect of all these ions on store-dependent Ca^{2+} influx, and thus reduced levels of Ca^{2+} within the intracellular stores. The ability of K^+ to enhance ADP-dependent Ca^{2+} release in the presence of 10 μM nifedipine (Fig. 4, A and B) also rules out a role for dihydropyridine receptors acting directly on G protein-coupled cascades, and thus IP_3 production, as shown to occur in skeletal and smooth muscle (Araya et al., 2003; Valle-Rodriguez et al., 2003). Several pieces of evidence, therefore, demonstrate that the effect of K^+ on $P2Y_1$ receptor-evoked Ca^{2+} responses depends not upon activation of voltage-gated Ca^{2+} channels or other forms of Ca^{2+} influx but upon the release of Ca^{2+} from internal stores.

Essential Role for Phospholipase-C, and Thus IP_3 Production, in the Responses to ADP and K^+. Pretreatment of 1321-N1-*h*$P2Y_1$ cells for 10 min with 10 μM U-73122, a phospholipase-C inhibitor (Smith et al., 1990), abolished the response to both ADP and ADP in high K^+ (Fig. 5). In contrast, an identical treatment with the inactive analog U-73343, had no significant effect on the $[Ca^{2+}]_i$ increases

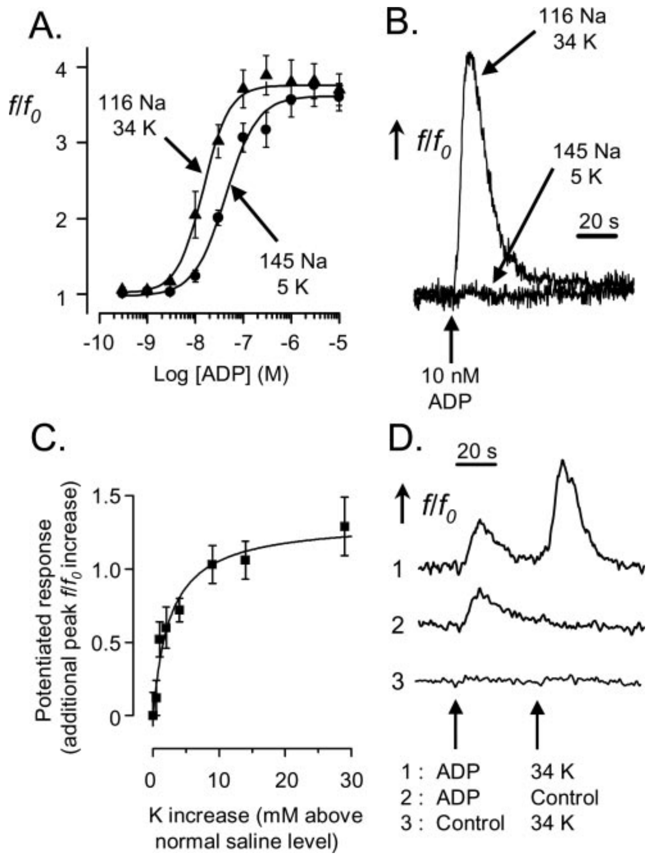

Fig. 2. Concentration dependence to the potentiation of $P2Y_1$ receptors by extracellular K^+. Average ADP- and K^+-evoked intracellular Ca^{2+} responses (fluo-4 f/f_0 ratio) measured in populations of 1321-N1-*h*$P2Y_1$ cells using a Flexstation II multiwell fluorimeter. A, peak $[Ca^{2+}]_i$ increase as a function of ADP concentration added in normal saline (5 mM K^+; circles) or high K^+ saline (final K^+, 34 mM; triangles). The data were fit to the equation $y = A/1 + (EC_{50}/x)^h$, where A is the maximal potentiated response, EC_{50} is the ADP concentration generating half-maximal potentiated response, and h is the slope. The average EC_{50} was 53 nM in 5 mM K^+ and 20 nM in 34 mM K^+. B, sample responses to 10 nM ADP added in normal and high K^+ saline, demonstrating the marked potentiation of $P2Y_1$ receptor responses by this cation at threshold levels of stimulation. C, relationship between extracellular K^+ increase (above the normal saline concentration of 5 mM) and the potentiated Ca^{2+} response to 20 nM ADP. The solid line was fit to the equation $y = Vx/K_m + x$, where V is the maximal potentiated response (1.32) and K_m the additional K^+ concentration that generates half the maximal potentiation (2.6 mM, thus a total saline K^+ of 7.6 mM). D, potentiation of $P2Y_1$ receptor-dependent Ca^{2+} responses by K^+ added after the initial agonist-evoked response. Two 50-μl additions were made (arrows) in each of the three recordings (1–3). The first addition was either 100 nM ADP (1 and 2) or normal saline without agonist (3). The second addition maintained the initial ADP concentration and either increased external K^+ from 5 to 34 mM (1 and 3) or maintained K^+ at 5 mM (control; 2).

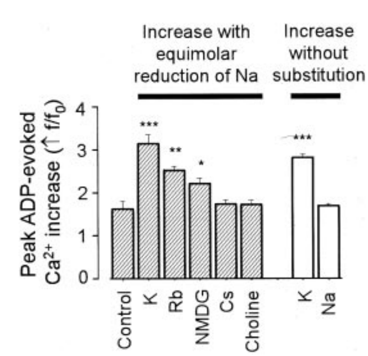

Fig. 3. Comparison of the ability of different monovalent cations to potentiate the $P2Y_1$ receptor. Comparison of peak $[Ca^{2+}]_i$ responses (fluo-4 f/f_0 ratio) to 100 nM ADP measured in 1321-N1 cells expressing *h*$P2Y_1$. Each monovalent cation was increased by 30 mM, with either an equal reduction in Na^+ (closed columns) in the control saline (normal saline; see *Materials and Methods*) or simply by addition to the normal saline (open columns). The responses in elevated K^+, Rb^+, and $NMDG^+$ were significantly different to control, whereas Cs^+, choline$^+$, and Na^+ had no significant effect.

evoked by ADP and ADP/high K^+ (Fig. 5C). This indicates an essential role for activation of phospholipase-C and thus IP_3 production in the response to K^+. The 1321-N1-P2Y_1 cells lacked functional Ca^{2+}-induced Ca^{2+} release via ryanodine receptors, because 10 mM caffeine failed to generate a Ca^{2+} response (data not shown). Thus, IP_3-dependent Ca^{2+} release can fully explain the response to ADP and ADP/K^+. This is consistent with previous studies in both heterologous and native systems demonstrating that the P2Y_1 receptor couples to Ca^{2+} mobilization via G_q proteins and phospholipase-Cβ (Nicholas et al., 1996; Offermanns et al., 1997; Martinez-Pinna et al., 2005) and suggests that K^+ directly enhances P2Y_1 receptor-dependent activation of this IP_3-generating pathway.

Role of Membrane Depolarization in the Potentiation of P2Y_1 Receptors by Extracellular K^+. One major effect of an increase in $[K^+]_o$ is membrane depolarization, which we have shown to directly enhance Ca^{2+} release evoked by ADP via P2Y_1 receptors in the megakaryocyte (Martinez-Pinna et al., 2005, and references therein). 1321-N1 cells readily form electrical connections with their neighbors; therefore, voltage-clamp experiments proved difficult, and we turned to "current-clamp" whole cell patch-clamp measurements combined with single cell photometry to assess the role of membrane potential in the $[Ca^{2+}]_i$ response to K^+. 1321-N1-*h*P2Y_1 cells held under patch clamp were generally less responsive to ADP compared with the noninvasive conditions used in Figs. 1 and 2, possibly because of mechanically triggered release of ATP/ADP during gigaOhm seal formation and thus partial receptor desensitization. For example, 100 nM ADP usually evoked only a small or negligible $[Ca^{2+}]_i$ increase (Fig. 6, A and B). Nevertheless, an increase in K^+ still caused a substantial $[Ca^{2+}]_i$ increase if applied in addition to the nucleotide (Fig. 6, A and B). For a $[K^+]_o$ increase of 30 mM, a substantial membrane depolarization (30 ± 5 mV; n = 5) was observed in parallel with the $[Ca^{2+}]_i$ increase. This is within the range of depo-

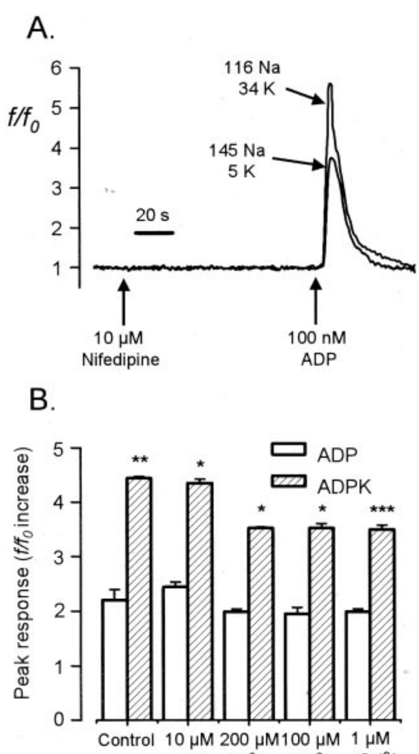

Fig. 4. Potentiation of P2Y_1 receptors by external K^+ is maintained in the presence of nifedipine or other inhibitors of voltage-gated Ca^{2+} channels. A, intracellular Ca^{2+} responses (f/f_0 ratios) of a semiconfluent monolayer of 1321-N1-*h*P2Y_1 cells to 100 nM ADP in saline containing 5 mM or 34 mM K^+ (145 and 116 mM Na^+, respectively), both in the presence of 10 μM nifedipine. B, comparison of the average peak Ca^{2+} increase (increase in f/f_0 response; n = 4) evoked by 100 nM ADP in normal saline (5 mM K^+, open columns) and high K^+ saline (34 mM K^+; closed columns) in the absence (control) and presence of either 10 μM nifedipine, 200 μM $NiCl_2$, 100 μM $CdCl_2$, or 1 μM $GdCl_3$.

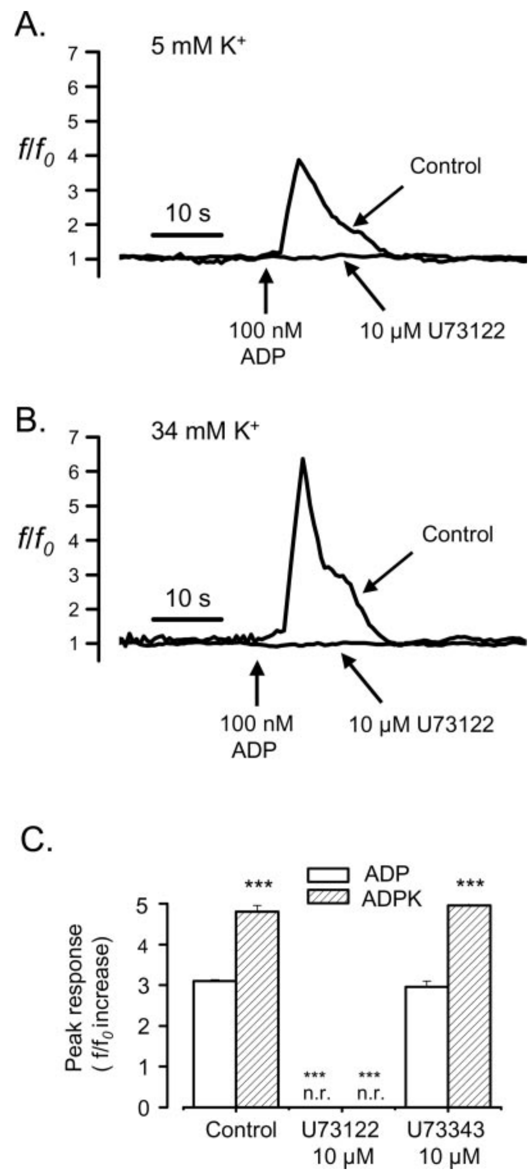

Fig. 5. Ca^{2+} signaling via P2Y_1 receptors at normal and elevated K^+ concentrations is entirely dependent on stimulation of phospholipase-C and thus IP_3 production. A and B, comparison of responses to 100 nM ADP in the absence (control) and the presence of the phospholipase C inhibitor U-73122 (10 μM; 10 min) in normal saline (5 mM K^+) (A) and high K^+ saline (34 mM K^+) (B). The $[Ca^{2+}]_i$ (fluo-4 f/f_0 ratio) was measured from a semiconfluent monolayer of 1321-N1-*h*P2Y_1 cells. C, comparison of the peak f/f_0 increases evoked by 100 nM ADP in 5 mM K^+ (open columns) and 34 mM K^+ (shaded columns) under control conditions or after a 10-min incubation with either U-73122 (10 μM) or its analog U-73343 (10 μM). The responses are the average of six experiments. There was no response (n.r.) in the presence of U-73122.

larizations previously reported to directly potentiate Ca^{2+} mobilization via $P2Y_1$ receptors in the electrically inexcitable megakaryocyte (Martinez-Pinna et al., 2004). A 1.5 mM K^+ increase was also able to mobilize Ca^{2+} during exposure of 1321-N1-*h*P2Y1 cells to 100 nM ADP, whereas the membrane potential displayed only a very small (≤3mV) depolarization. Spontaneous depolarizations of similar or slightly larger amplitude were observed in many cells during exposure to ADP alone without inducing changes in $[Ca^{2+}]_i$ (see, for example, Fig. 6B). This suggests that K^+ potentiates $P2Y_1$ receptor signaling in part independently of membrane potential shifts. The megakaryocyte is a cell type in which the ADP-evoked $[Ca^{2+}]_i$ response depends upon $P2Y_1$ receptors (Martinez-Pinna et al., 2005) and is amenable to whole cell voltage-clamp recordings without incurring major P2Y receptor desensitization (Martinez-Pinna et al., 2004). Application of 30 nM ADP to a megakaryocyte clamped at −75 mV generated a small oscillatory $[Ca^{2+}]_i$ increase followed by a sustained plateau phase (Fig. 6C). Subsequent elevation of $[K^+]_o$ from 5 to 35 mM without alteration of the membrane potential generated a large $[Ca^{2+}]_i$ transient. This effect was also observed in Ca^{2+}-free saline ($n = 5$; not shown), confirming that K^+ enhances ADP-evoked Ca^{2+} release. Thus, K^+ is able to potentiate $P2Y_1$ receptor-evoked Ca^{2+} mobilization by both voltage-dependent (Martinez-Pinna et al., 2004) and voltage-independent mechanisms.

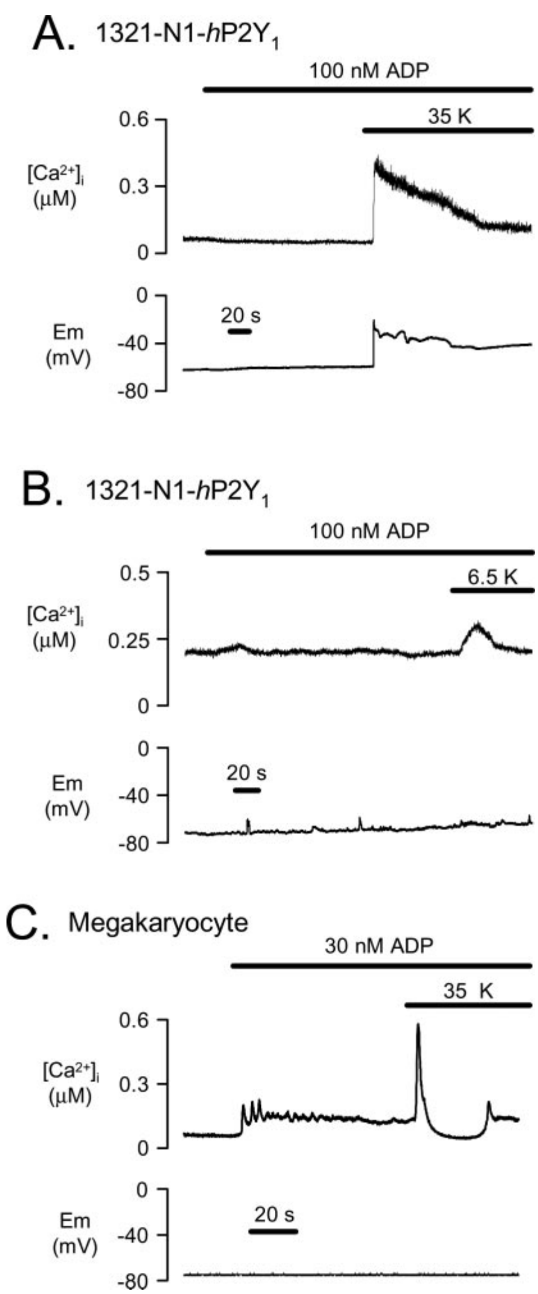

Fig. 6. Role of membrane potential in the K^+-dependent potentiation of $P2Y_1$ receptors. A and B, simultaneous membrane potential and intracellular Ca^{2+} recordings in 1321-N1-*h*$P2Y_1$ cells during application of 100 nM ADP and during elevation of external K^+ from 5 mM to either 35 mM (A) or 6.5 mM (B). C, effect of 30 nM ADP with a subsequent increase in external K^+ concentration from 5 to 35 mM in a rat megakaryocyte held under voltage clamp at −75 mV. Recordings are representative of at least five cells.

Extracellular K^+ Potentiates Ca^{2+} Mobilization Stimulated by Other P2Y Receptors. To investigate whether other P2Y receptor subtypes are modulated by K^+, we turned to HEK 293 cells, which display robust endogenous Ca^{2+} responses to UTP. UTP potently stimulates IP_3-dependent Ca^{2+} mobilization via $P2Y_2$ and $P2Y_4$, but not $P2Y_1$ receptors (Nicholas et al., 1996; Leon et al., 1997). RT-PCR revealed the presence of transcripts for $P2Y_4$ (Fig. 7A), but not $P2Y_2$, in the HEK 293 cells used for the present study. This is consistent with a previous quantitative mRNA study on HEK 293 cells (Moore et al., 2001) and with evidence from immunocytochemical and functional studies (Fischer et al., 2003; Wirkner et al., 2004) that the $P2Y_4$ mRNA there encodes the $P2Y_4$ receptor (Moore et al., 2001). An elevation of external K^+ in the absence of agonist activated a $[Ca^{2+}]_i$ increase because of the presence of endogenous voltage-gated Ca^{2+} channels (Berjukow et al., 1996), which was entirely blocked by 200 μM $NiCl_2$ (Fig. 7B). However, even in the presence of 200 μM Ni^{2+}, K^+ was able to potentiate the $[Ca^{2+}]_i$ response to UTP when added simultaneously with the agonist (particularly at threshold levels of the agonist; Fig. 7C), or if added subsequent to the initial UTP-evoked Ca^{2+} transient (Fig. 7D, trace 1). As observed for $P2Y_1$-evoked Ca^{2+} responses, the effect of K^+ on the initial response to UTP was caused by a leftward shift in the concentration-response curve without a change in the slope (Fig. 7E). The EC_{50} shifted 10-fold from 30 ± 4 μM in 5 mM K^+ to 2.8 ± 3 μM in 34 mM K^+ ($n = 4$; $p < 0.01$), which was more pronounced than the effect on ADP stimulation of 1321-N1-*h*$P2Y_1$ cells (see above). Furthermore, K^+ produced a significant increase in the maximal response to UTP (Fig. 7E), which was not observed for $P2Y_1$ receptors. Together, these data indicate that $P2Y_4$, like $P2Y_1$ receptors are potentiated by increases in extracellular K^+ and that this may be a common feature of G protein-coupled nucleotide receptors.

Discussion

The $P2Y_1$ receptor displays a very widespread distribution in adult and developing tissues (Simon et al., 1997; Moore et al., 2001; Cheung et al., 2003). This identified subtype has well established roles in hemostasis and thrombosis (Kunapuli et al., 2003), and evidence is emerging for specific functions in other tissues such as the regulation of gene expression of some synaptic effectors in skeletal muscle (Tsim et al., 2003) and modulation of some neuronal ion channels (Filip-

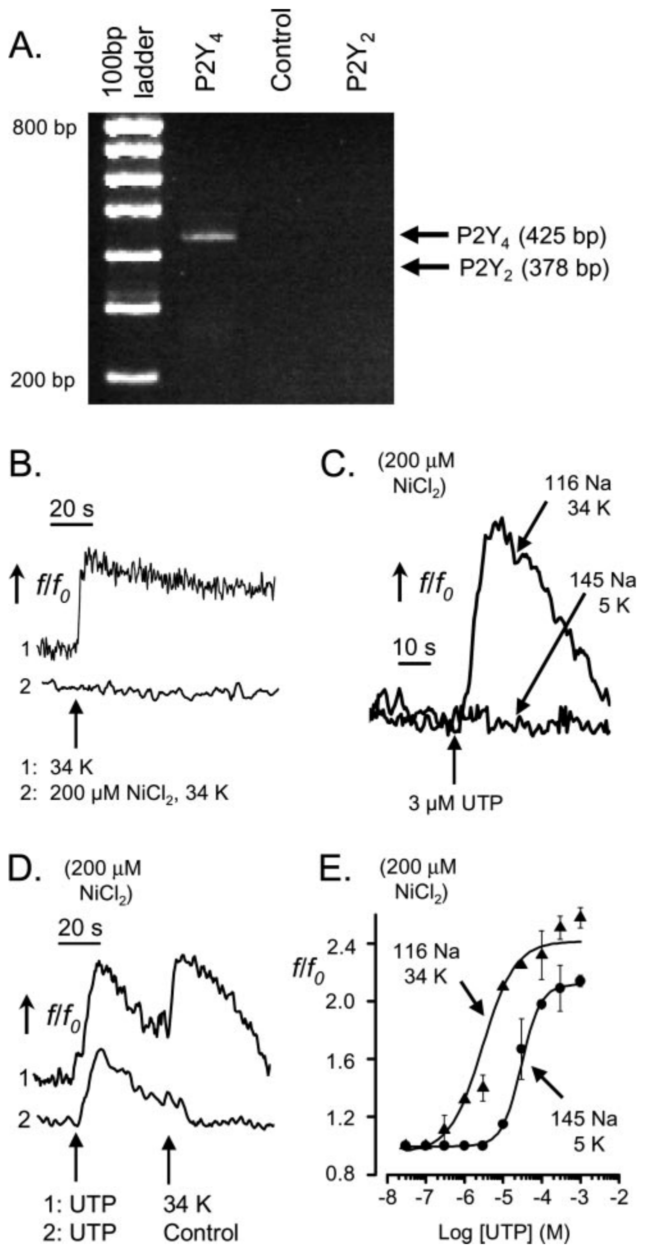

Fig. 7. K^+ potentiates endogenous UTP-dependent Ca^{2+} responses in HEK 293 cells. A, RT-PCR products for $P2Y_2$ and $P2Y_4$ receptor subtypes in HEK 293 cells. The arrows indicate expected amplicons for $P2Y_4$ (425 bp) and $P2Y_2$ (378 bp). The control lane shows a sample treated as for detection of $P2Y_4$ but without reverse transcriptase. The samples were run on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide. B to E, population $[Ca^{2+}]_i$ responses (fluo-4 f/f_0 ratio) in semiconfluent monolayers of nontransfected HEK 293 cells. B, response to a 29 mM elevation of external K^+ (5 to 34 mM K^+, with an equimolar reduction of Na^+) in normal saline (trace 1) and in cells pretreated for 1 min with 200 μM $NiCl_2$ (trace 2). All experiments in C to E were conducted in the presence of 200 μM $NiCl_2$. C, response to a threshold concentration of UTP (3 μM) in normal and high K^+ salines (5 and 34 mM K^+, respectively). D, effect of elevating K^+ (5 to 34 mM, trace 1) after initially stimulating with 30 μM UTP. Trace 2 shows the control response in which the second addition was an equal volume of normal saline. The second additions did not change the concentration of UTP. Traces are representative of four experiments. E, peak $[Ca^{2+}]_i$ increase as a function of UTP concentration added in normal saline (5 mM K^+; circles) or high K^+ saline (final K^+, 34 mM; triangles). The data were fitted to the equation $y = A/1 + (EC_{50}/x)^h$, where A is the maximal potentiated response, EC_{50} is the ADP concentration generating half-maximal potentiated response, and h is the slope. The average EC_{50} was 30 μM in 5 mM K^+ and 2.8 μM in 34 mM K^+.

pov et al., 2004). We now show that physiologically relevant increases in $[K^+]_o$ significantly potentiate signaling via $P2Y_1$ receptors via a mechanism independent of calcium influx or voltage-gated calcium channels. The synergy that we demonstrate could amplify cellular responses in a number of situations. For example, tissue injury will extensively release K^+ from the cytoplasm of damaged cells and may act to accelerate the initial stages of hemostasis by potentiation of platelet $P2Y_1$ receptor responses (Kunapuli et al., 2003). Furthermore, concurrent extracellular increases in both ATP and K^+ can occur. For example, in skeletal muscle, ATP is released either with acetylcholine at the neuromuscular junction or from muscle fibers passively when stressed (Schwiebert and Zsembery, 2003) and significant $[K^+]_o$ increases are known to occur in this tissue, particularly during exercise. Therefore, the effect we observe can be postulated as a link between levels of activity and gene expression or other signaling events in skeletal muscle. Venous K^+ can increase to ≈6 mM in moderate exercise and to almost 10 mM during periods of extreme physical activity (Sejersted and Sjøgaard, 2000). $P2Y_1$ receptors are located in several cardiovascular tissues, including the heart, endothelium, and vascular smooth muscle; the effect we observe here could therefore play a widespread role in adaptations to exercise. $P2Y_1$ receptors are also located in sensory ganglia (Ruan and Burnstock, 2003), and both noxious stimuli and painful injury are known to evoke a sustained increase in $[K^+]_o$ of up to 3 mM (Svoboda et al., 1988). Therefore, the K^+ dependence of the $P2Y_1$ receptor [or other P2Y subtypes expressed in these ganglia (Moriyama et al., 2003) and exhibiting a dependence upon $[K^+]_o$] could have relevance to the mechanisms underlying neuropathic pain.

Potentiation of GPCR responses by K^+ is not confined to $P2Y_1$ receptors, because a similar effect was observed for UTP-dependent Ca^{2+} mobilization involving endogenous $P2Y_4$ receptors in HEK 293 cells (Fig. 7). In fact, K^+ caused an even greater leftward shift in the dose-response curve for this UTP response compared with ADP activation of $P2Y_1$ receptors (10-fold compared with ≈3-fold for $P2Y_1$). K^+ also enhanced the maximal response to UTP, which was not significantly observed in 1321-N1-hP2Y$_1$ cells and may reflect a greater overall level of amplification for $P2Y_4$ compared with $P2Y_1$ receptors. UTP-sensitive $P2Y_4$ receptors are expressed on a range of neuronal cell types (Ruan and Burnstock, 2003). In addition, $P2Y_4$ has been shown to be expressed on many epithelial surfaces (Suarez-Huerta et al., 2001; Unwin et al., 2003), where large ionic fluxes occur and thus where an elevation of K^+ may exert an important regulatory role.

It is well established that small increases in the extracellular K^+ concentration, similar to those that we show potentiate P2Y receptors, can generate large increases in intracellular Ca^{2+} in the adrenal glomerulosa cell (Spat and Hunyady, 2004). The increase in $[Ca^{2+}]_i$ leads to release of aldosterone and thus physiological responses to regulate plasma K^+ levels. However, the mechanism underlying the response to K^+ in the glomerulosa cell contrasts with the effect on P2Y receptors in that it depends upon activation of Ca^{2+} influx via T-type Ca^{2+} channels. Synergy is observed between elevated K^+ and angiotensin II, although the underlying mechanism is again caused by effects on voltage-gated Ca^{2+} influx (Spat and Hunyady, 2004).

As a consequence of the leftward shift in the ADP or UTP concentration-response curve, the amplification was particularly pronounced when K^+ was added simultaneously with ADP at near-threshold levels of the agonist (Figs. 1F, 2B, and 7B). It was also interesting to note that K^+ generated substantial $[Ca^{2+}]_i$ increases if added after the initial agonist-evoked transient, when the $[Ca^{2+}]_i$ had returned to near resting levels (Figs. 1 and 2D). Furthermore, at the single cell level, the effect of a K^+ increase of only 1.5 mM subsequent to the agonist (Fig. 1, E and F) produced an initial $[Ca^{2+}]_i$ spike of similar amplitude to that generated by a much higher K^+ level (30 mM increase; Fig. 1B). In part, this may reflect the nonlinear highly cooperative nature of the IP_3-dependent Ca^{2+} release from stores (Meyer et al., 1988); however, it may also reflect an ability of K^+ to act more effectively on an agonist-bound receptor state.

We have previously shown that Ca^{2+} signaling via $P2Y_1$ receptors in the megakaryocyte is markedly potentiated by membrane depolarization (Martinez-Pinna et al., 2005). The response is graded with depolarizing pulse amplitude without evidence for a threshold potential (Martinez-Pinna et al., 2004). Thus, the potentiation of $P2Y_1$ receptors by a 30 mM K^+ increase, which depolarized the 1321-N1 cells by ≈30 mV, could in part involve a direct effect of membrane potential. However, an increase of only 1.5 mM K^+, which is effective at enhancing $P2Y_1$ receptors in 1321-N1 cells (Fig. 1E), predictably had negligible effects on the membrane potential. In addition, K^+ potentiated the ADP (1 μM)-evoked $[Ca^{2+}]_i$ increase at a constant membrane potential in rat megakaryocytes (Fig. 6), a native cell type where this response is dependent upon the presence of $P2Y_1$ receptors (Martinez-Pinna et al., 2005). Thus, K^+ enhances $P2Y_1$ receptor signals via both membrane depolarization and via a more direct effect.

Regarding the underlying mechanism, the complete block of agonist-induced calcium responses by U-73122 in both control cells and cells exposed to elevated K^+ is consistent with an effect of the cation at the receptor level leading to calcium mobilization via an IP_3-dependent pathway. We can exclude effects of K^+ in the range studied here on the relative amounts of the forms of ADP in solution. ADP is largely complexed there with divalent cations as a result of its high affinity for Mg^{2+} and Ca^{2+} compared with K^+ (stability constants for Mg^{2+}, Ca^{2+}, and K^+ binding to ADP have been reported to be 3, 2.81, and 0.67, respectively; Sillen and Martell, 1971). Furthermore, the stability constants for Na^+ and K^+ are virtually identical (0.65 and 0.67, respectively; Sillen and Martell, 1971); therefore, the standard experimental protocol used in this study, involving equimolar reduction in Na^+ with elevation of K^+, will not alter the level of free ADP. Indeed, the only changes will be extremely small increases in $KADP^{2-}$ and decreases in $NaADP^{2-}$. A reasonable explanation for the voltage-independent effect of K^+ on $P2Y_1$ receptors would be allosteric binding to one or more sites on the exofacial surface. A precedent for such a monovalent cation binding exists in the well established allosteric modulation by intracellular Na^+ of several G protein-coupled receptors via binding to a site containing a critical aspartate residue (Horstman et al., 1990). In the present study, the half-maximal value of the K^+ concentration dependence was 4.2 or 7.6 mM, for starting concentrations of 0 and 5 mM, respectively, and gives an estimate of the operative K^+ affinity. The affinities for K^+ and Na^+ on many proteins are generally a hundredfold or higher, suggesting a specific K^+ binding site at the $P2Y_1$ receptor, as found in a few other well established examples where K^+ is functional. For example, the K^+ affinity in *Shaker* K^+ channels has been estimated (Thompson and Begenisich, 2001) at 2.7 mM for its high-affinity state when one K^+ is in the pore, weakened (allowing fast ion flow) to 65 mM when two ions are there, because of their mutual repulsion and a conformational change (Zhou and MacKinnon, 2003). This K^+-chelating site is built from a serine OH and four backbone carbonyls (Zhou and MacKinnon, 2003). A few enzymic proteins also bind an essential K^+ ion, some decarboxylases (Toney et al., 1995) and tryptophanase (Isupov et al., 1998), the latter having affinity of 1.4 mM and using, rather similarly, a Glu carboxylate oxygen and four backbone carbonyls. Hence, a K^+ binding site on the $P2Y_1$ receptor would be within the known range of functional K^+-protein interactions.

In conclusion, we show for the first time that physiologically relevant increases in extracellular K^+ significantly potentiate signaling via P2Y receptors. Depolarization can account for part of the response at high, pathophysiological levels of K^+; however, the cation also potentiates $P2Y_1$ receptors independently of a change in membrane potential.

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