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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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Abbreviations: $[K^+]_o$, extracellular K^+ concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GPCR, G-protein-coupled receptor; FBS, fetal bovine serum; PSA, penicillin, streptomycin and amphotericin; DMEM, Dulbecco's modified Eagle's medium; NMDG, n-methyl-D-glucamine; IP_3, D-*myo*-Inositol 1,4,5-Trisphosphate

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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 U73122, but not by its analogue U73343, nor by nifedipine, Ni^{2+}, Cd^{2+} or Gd^{3+} Thus, K^+ enhances IP_3-dependent Ca^{2+} release without a requirement for Ca^{2+} influx. The cation-dependence of this effect displayed the order K^+>Rb^+>$NMDG^+$, with Cs^+ and $Choline^+$ being ineffective. The potentiation by K^+ increase 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 (Martinez-Pinna et al. 2004 J.Physiol. **555,** 61-70). 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 HEK 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.

INTRODUCTION

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 Sjogaard, 2000). In addition, cellular damage or ischaemia 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 the case of the 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 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 are involved in the regulation of a wide range of physiological processes. Their activation mechanism is not normally considered to be

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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 (Sejersted and Sjogaard, 2000;Sykova, 1983), 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.

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EXPERIMENTAL PROCEDURES

Solutions and Reagents. The standard external saline contained (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$ 10 HEPES, 1 $CaCl_2$, 10 D-glucose (pH 7.35 with NaOH). For Na^+-free saline, NaCl was replaced by an equal concentration of CholineCl. Elevation of K^+ or other cations was by equimolar substitution of the Cl^- salt for NaCl (or CholineCl), except for the experiment shown by the open bar 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 (in mM) 150 KCl, 2 $MgCl_2$, 0.1 EGTA, 10 HEPES, 0.05 K_5Fura-2, 0.05 Na_2GTP (pH 7.2 with KOH). Dulbecco's modified Eagle's medium (DMEM) and Geneticin were from Invitrogen (Paisley, UK). K_5fura-2, fura-2AM, fluo-3AM and fluo-4AM were from Molecular Probes (Leiden, Netherlands). All other reagents, were purchased from Sigma (Poole, Dorset, UK). ADP and 2MeSADP (Sigma, UK) were treated by incubation with hexokinase and glucose, and ATP and 2MeSATP were treated with creatine phosphate/creatine phosphokinase, to remove contaminating triphosphate or diphosphate nucleotides respectively, as described previously (Tung *et al.*, 2004;Mahaut-Smith *et al.*, 2000).

Cell preparation. 1321-N1 astrocyoma 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 (FBS), 1 % penicillin, streptomycin and amphotericin antibiotic antimycotic (PSA) solution, and 600 µg/ml Geneticin 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 due to activation only of $P2Y_1$ receptors. Firstly, the

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order of efficacy in $[Ca^{2+}]_i$ responses was 2MeSADP>ADP>2MeSATP>ATP, that being the known agonist profile of the $P2Y_1$ receptor (Leon *et al.*, 1997;Nicholas *et al.*, 1996). Secondly, the $P2Y_1$ receptor-specific antagonist MRS 2179 competitively inhibited the ADP-evoked Ca^{2+} response. Thirdly, 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 % FBS and 1 % PSA solution. Megakaryocytes from the femoral and tibial marrow of adult male Wistar rats were prepared for whole cell patch clamp as described in detail elsewhere (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 microtitre plates (Appleton Woods, Selly Oak, Birmingham, UK). Cells were loaded with fluo-4 by incubation with 2 μM fluo-4 AM 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 longpass filter. At the start of each experiment, the cells were bathed in either 200 or 150 μl 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

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were grown on glass coverslips to ≥60 % confluency. 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 performed using a Cairn spectrophotometer system (Cairn Research Ltd, Kent UK), during simultaneous whole cell patch clamp, as described in detail elsewhere (Martinez-Pinna *et al.*, 2004). Fluorescence imaging was performed on a Zeiss LSM 510 confocal microscope (Carl Zeiss Ltd, 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 – 30 cells at a rate of 0.5 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 3-3.5 MΩ. Megakaryocytes were held under voltage clamp, as previously described (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 Ltd, UK) and cDNA prepared using the Omniscript® RT Kit (Qiagen Ltd, UK). Forward and reverse oligonucleotide primers were as described elsewhere (Jin *et al.*, 1998). After initial denaturation for 135 sec at 95°C, 35 PCR cycles with 5 U/μl *Taq* polymerase (Qiagen Ltd, UK) were conducted as follows: denaturation at 95°C for 40 sec, annealing at 65°C ($P2Y_4$) or 55°C ($P2Y_2$) for 40 sec and extension at 72°C for 40 sec, followed by 10 min at 72°C. Controls to verify that amplified products

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were not derived from genomic DNA omitted the reverse transcriptase during the RT step, but were otherwise identical.

Data manipulation and statistics. Experiments shown for single cell recordings are representative of at least 5 other cells. Fluo-4 and fluo-3 fluorescence signals (f) were expressed as f/f_0 ratios to normalise 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^{o}C). Data were exported for analysis and fitting of concentration response relationships within Microcal Origin version 6.0 (Microcal software Inc, Northampton, MA, USA). 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 0.05 (*), 0.01 (**) or 0.005 (***).

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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 intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by a constant $[Ca^{2+}]_i$ indistinguishable from that of the resting state. Fig. 1A shows an example of the response at the single cell level measured by fluorescence imaging. An increase in $[K^+]_o$ of 30 mM (from 5 to 35 mM, with equimolar reduction in Na^+) 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 due to the increase in K^+ and not the simultaneous decrease in Na^+ as no change in $[Ca^{2+}]_i$ was observed if 30 mM Na^+ was replaced by $Choline^+$ (not shown, but see section below comparing different monovalent cations). 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 an increase in $[K^+]_o$ (from 5 to 35 mM) 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^+, since the K^+-dependence of this pump is saturated at a $[K^+]_o$ of 5 mM under Na^+-free conditions (Glynn *et al.*, 1970). Importantly, enhancement of P2Y$_1$ responses was observed following smaller increases in $[K^+]_o$, even from 5 to 6.5 mM (Fig. 1E), which is equivalent to the shift in $[K^+]_o$ that has been estimated to occur in skeletal

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muscle T-tubules under physiological conditions (Sejersted and Sjogaard, 2000). A 1.5 mM increase in $[K^+]_o$ evoked a single $[Ca^{2+}]_i$ transient whereas the response to 30 mM was more robust, often causing multiple Ca^{2+} spikes (compare Figs 1B & E). However, due to 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 Sjogaard, 2000).

Extracellular K^+ decreases the EC_{50} for ADP at the $P2Y_1$ receptor

To further characterise 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

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normal saline following 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. 2A-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 (see Fig. 1). The lack of effect of saline addition after 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 1-10 mM caused a concentration-dependent decrease in ADP-evoked Ca^{2+} responses (not shown) as previously reported 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-*h*$P2Y_1$ cell (Fig. 3). The ability to enhance the initial Ca^{2+} increase evoked by 100 nM ADP displayed the order of potency: $K^+ > Rb^+ > NMDG^+$, while 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

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enhancement of ADP-mediated Ca^{2+} responses when K^+ was increased without altering the saline Na^+ concentration (Fig. 3, open bar).

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 1-2 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+} (200μM), 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^+ enhances 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 to 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

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intracellular stores. The ability of K^+ to enhance ADP-dependent Ca^{2+} release in the presence of 10 μM nifedipine (Fig. 4A,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 does not depend upon activation of voltage-gated Ca^{2+} channels or other forms of Ca^{2+} influx, but is due to release of Ca^{2+} from internal stores.

Essential role for phospholipase-C, and thus IP_3 production, in the responses to ADP and K^+

Pre-treatment of 1321-N1-*h*$P2Y_1$ cells for 10 min with 10 μM U73122, a phospholipase-C inhibitor (Smith *et al.*, 1990), totally abolished the response to both ADP and ADP in high K^+ (Fig. 5). In contrast, an identical treatment with the inactive analogue U73343, had no significant effect on the $[Ca^{2+}]_i$ increases 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 (CICR) via ryanodine receptors, as 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 (Martinez-Pinna *et al.*, 2005;Offermanns *et al.*, 1997;Nicholas *et al.*, 1996) and suggests that K^+ directly enhances $P2Y_1$ receptor-dependent activation of this IP_3-generating pathway.

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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 (see (Martinez-Pinna *et al.*, 2005) and references therein). 1321-N1 cells readily form electrical connections with their neighbours, 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 to the non-invasive conditions used in Figs. 1 and 2, possibly due to mechanically triggered release of ATP/ADP during gigaseal formation and thus partial receptor desensitisation. For example, 100 nM ADP usually evoked only a small or negligible $[Ca^{2+}]_i$ increase (Fig. 6A,B). Nevertheless, an increase in K^+ still caused a substantial $[Ca^{2+}]_i$ increase if applied in addition to the nucleotide (Fig. 6A, 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 depolarizations 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, while 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

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$[Ca^{2+}]_i$ (see for example Fig. 6B). This suggests that K^+ potentiates $P2Y_1$ receptor signalling 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 desensitisation (Martinez-Pinna *et al.*, 2004). Application of 30 nM ADP to a megakaryocyte clamped at –75mV generated a small oscillatory $[Ca^{2+}]_i$ increase followed by a sustained plateau phase (Fig. 6C). Subsequent elevation of $[K^+]_o$ from 5 mM 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.

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 (Leon *et al.*, 1997;Nicholas *et al.*, 1996). 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 expresses the $P2Y_4$ receptor.(Moore *et al.*, 2001) An elevation of external K^+ in the absence of agonist

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activated a $[Ca^{2+}]_i$ increase due to 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 due to 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.

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DISCUSSION

The $P2Y_1$ receptor displays a very widespread distribution in adult and developing tissues (Cheung *et al.*, 2003;Moore *et al.*, 2001;Simon *et al.*, 1997). This identified subtype has well established roles in haemostasis 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 (Filippov *et al.*, 2004). We now show that physiologically relevant increases in $[K^+]_o$ significantly potentiate signalling via $P2Y_1$ receptors and that this mechanism is independent of any contribution from 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 Sjogaard, 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

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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^+) could have relevance to the mechanisms underlying neuropathic pain.

Potentiation of GPCR responses by K^+ is not confined to $P2Y_1$ receptors, as 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 to ADP activation of $P2Y_1$ receptors (10 fold compared to ≈3-fold for $P2Y_1$). K^+ also enhanced the maximal response to UTP, which was not significantly observed in 1321-N1-*h*$P2Y_1$ cells and may reflect a greater overall level of amplification for $P2Y_4$ compared to $P2Y_1$ 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^+

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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 due to 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). 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, 2D). Furthermore, at the single cell level, the effect of a K^+ increase of only 1.5 mM subsequent to the agonist (Figs. 1E, F) produced an initial $[Ca^{2+}]_i$ spike of similar amplitude to that generated by a much higher K^+ level (30 mM increase, see Fig. 1B). In part this may reflect the non-linear highly co-operative 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+} signalling 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

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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. Additionally, K^+ potentiated the ADP (1μM)-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 U73122 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 to 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 of

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intracellular Na^+ on 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 zero and 5 mM respectively, and gives an estimate of the operative K^+ affinity. The affinities for K^+ and Na^+ on many proteins is generally a hundred or more fold higher, and suggests 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, due to their mutual repulsion and a conformational change (Zhou and MacKinnon, 2003). This K^+ chelating site is built from a serine OH and 4 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 4 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 signalling 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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FIGURE LEGENDS

Figure 1. Potentiation of $P2Y_1$ receptor-evoked intracellular Ca^{2+} responses by extracellular K^+ in single 1321-N1 cells. Each panel (**A-F**) shows the intracellular Ca^{2+} response from a single 1321-N1-$P2Y_1$ cell representative of 15-30 cells within a semi-confluent 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,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,F) with equimolar reduction of Na^+ (A-C; E,F) or choline (D). All salines contained 1 mM external Ca^{2+} except in C, which was nominally Ca^{2+}-free throughout. **A-E** show typical responses to increased saline K^+ concentration in cells which responded to ADP. Panel **F** shows the typical response for a cell that failed to respond to ADP alone, but then responded to increased K^+.

Figure 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 multi-well 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

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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, 2**), or normal saline without agonist (**3**). The second addition maintained the initial ADP concentration and either increased external K^+ from 5 mM to 34 mM (**1, 3**) or maintained K^+ at 5 mM (control, **2**).

Figure 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^+ (shaded bars) in the control saline (normal saline, see methods) or no reduction in Na^+ (open bar). The responses in elevated K^+, Rb^+ and $NMDG^+$ were significantly different to control, whereas Cs^+ and $Choline^+$ had no significant effect.

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Figure 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 semi-confluent 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 bars) and high K^+ saline (34 mM K^+, shaded bars) in the absence (control) and presence of either 10 μM nifedpine, 200 μM $NiCl_2$, 100 μM $CdCl_2$ or 1 μM $GdCl_3$.

Figure 5. Ca^{2+} signalling via $P2Y_1$ receptors at normal and elevated K^+ concentrations is entirely dependent on stimulation of phospholipase-C and thus IP_3 production. A,B. Comparison of responses to 100 nM ADP in the absence (control) and the presence of the phospholipase-C inhibitor U73122 (10 μM, 10 min) in **A.** normal saline (5 mM K^+) and **B.** high K^+ saline (34 mM K^+). The $[Ca^{2+}]_i$ (fluo-4 f/f_0 ratio) was measured from a semi-confluent 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 bars) and 34 mM K^+ (shaded bars) under control conditions or following a 10 min incubation with either U73122 (10 μM) or its analogue U73343 (10 μM). The responses are the average of 6 experiments. There was no response (n.r.) in the presence of U73122.

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Figure 6. Role of membrane potential in the K^+-dependent potentiation of $P2Y_1$ receptors. A,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 mM to 35 mM in a rat megakaryocyte held under voltage clamp at –75 mV. Recordings are representative of at least 5 cells.

Figure 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-E.** Population $[Ca^{2+}]_i$ responses (fluo-4 f/f_0 ratio) in semi-confluent monolayers of non-transfected HEK 293 cells. **B.** Response to an elevation of external K^+ (from 5 to 34 mM K^+, with an equimolar reduction of Na^+) in normal saline (trace 1) and in cells pre-treated for 1 min with 200 μM $NiCl_2$ (trace 2). All experiments in **C-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^+ (from 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 4 experiments. **E.** Peak $[Ca^{2+}]_i$ increase as a function of UTP concentration added in normal

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saline (5mM 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 5mM K^+ and 2.8 μM in 34 mM K^+.

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Figure 1

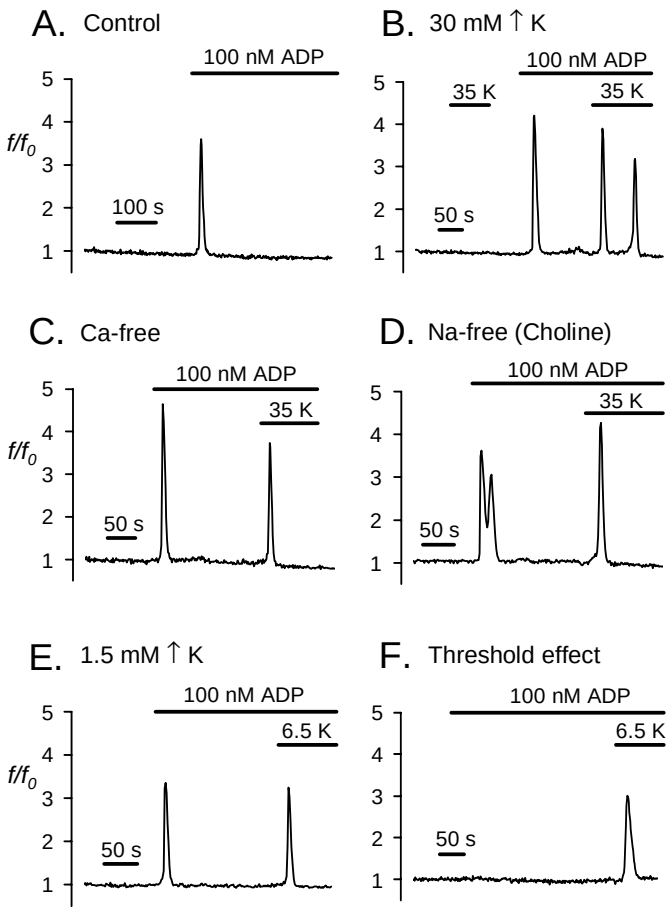

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Figure 2

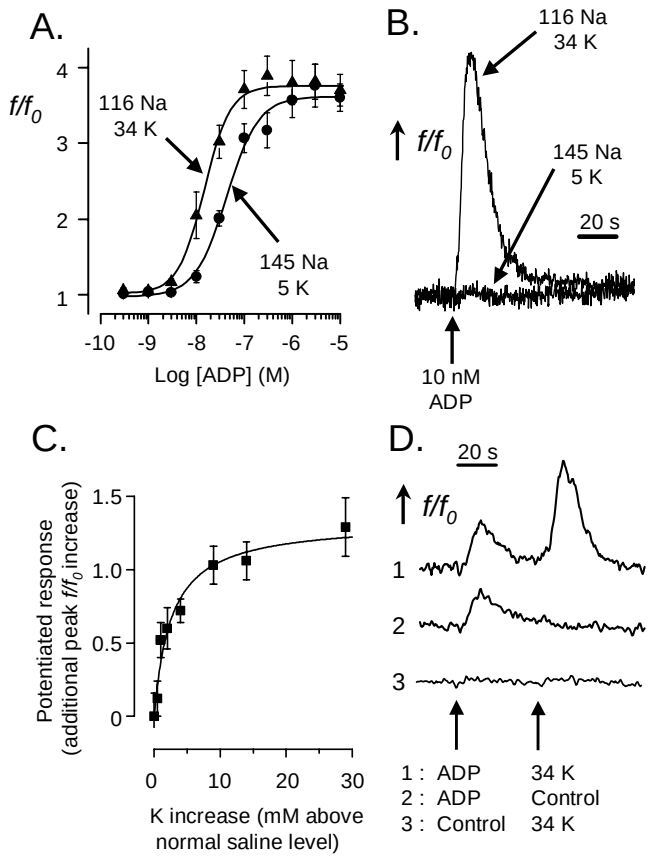

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Figure 3

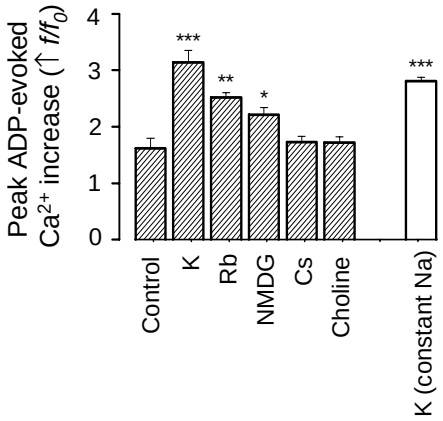

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Figure 4

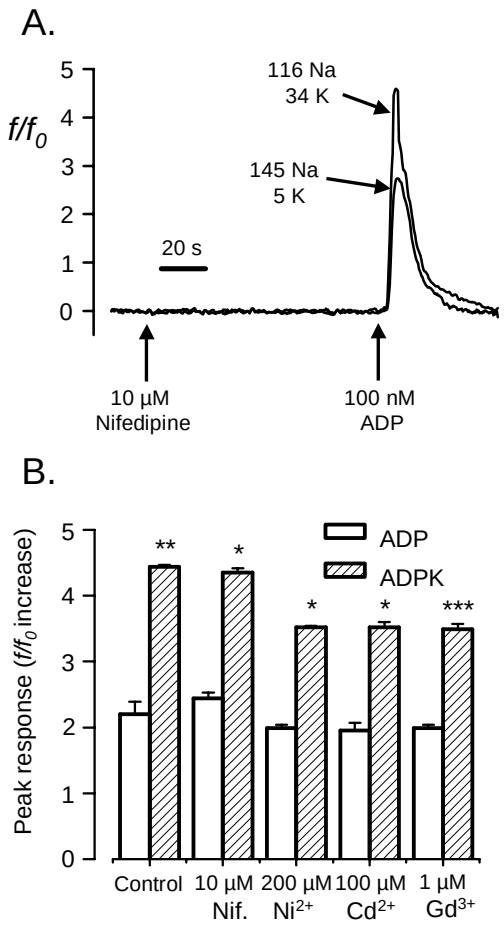

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Figure 5

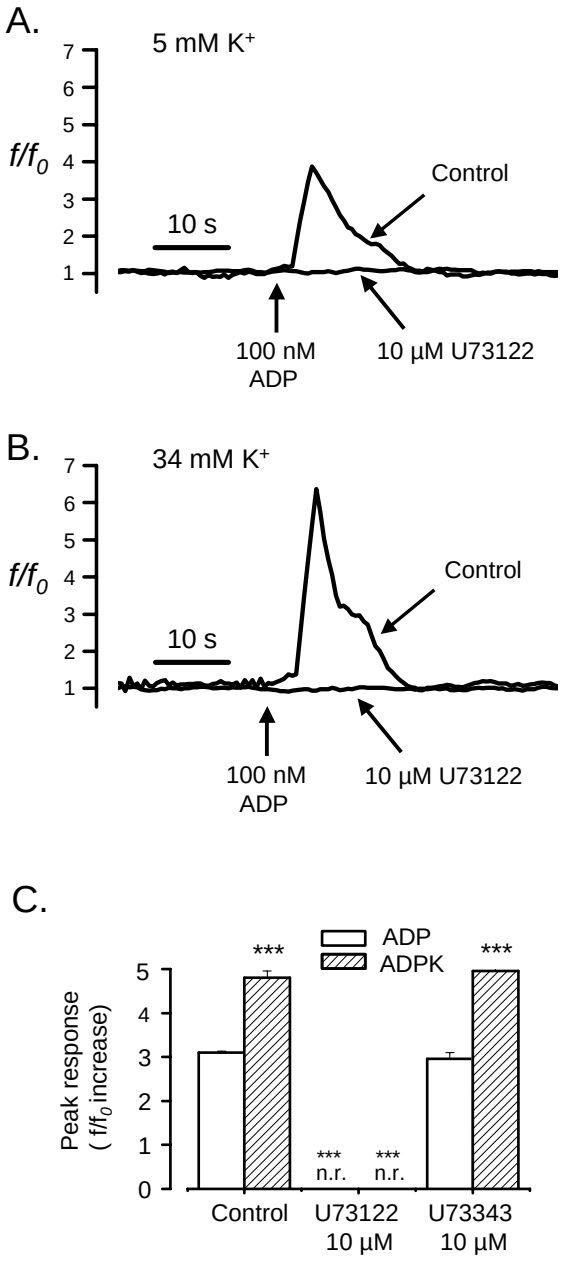

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Figure 6

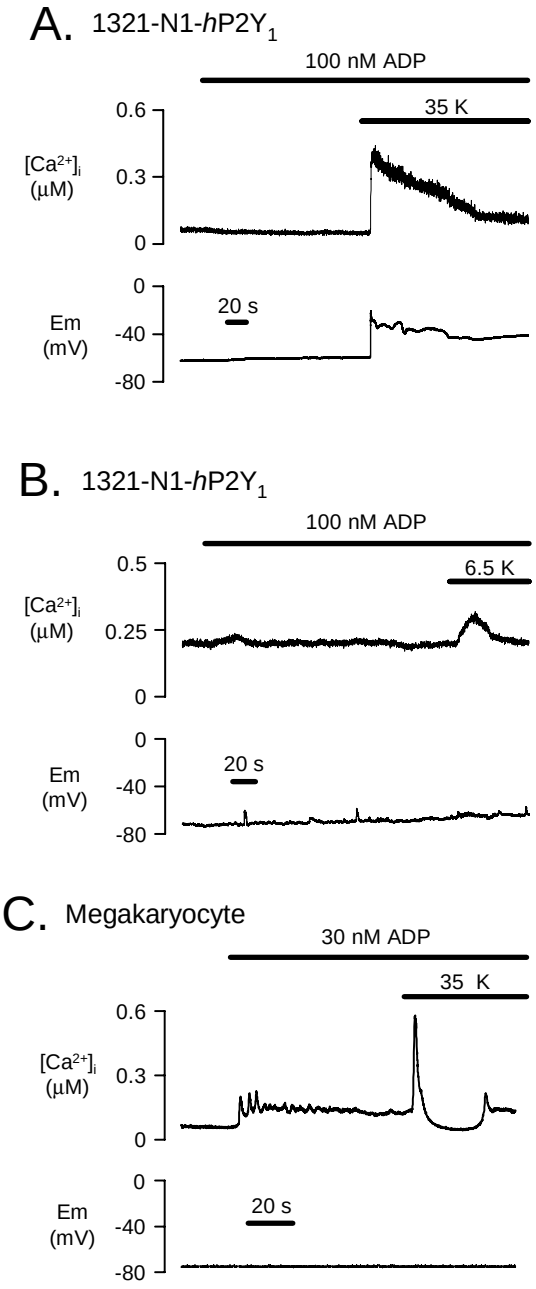

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Figure 7