Sensitivity limits for voltage control of P2Y receptor-evoked Ca^{2+} mobilization in the rat megakaryocyte

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G-protein-coupled receptor signalling has been suggested to be voltage dependent in a number of cell types; however, the limits of sensitivity of this potentially important phenomenon are unknown. Using the non-excitable rat megakaryocyte as a model system, we now show that P2Y receptor-evoked Ca^{2+} mobilization is controlled by membrane voltage in a graded and bipolar manner without evidence for a discrete threshold potential. Throughout the range of potentials studied, the peak increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to depolarization was always larger than the maximal reduction in $[Ca^{2+}]_i$ following an equivalent amplitude hyperpolarization. Significant $[Ca^{2+}]_i$ increases were observed in response to small amplitude (<5 mV, 5 s duration) or short duration (25 ms, 135 mV) depolarizations. Individual cardiac action potential waveforms were also able to repeatedly potentiate P2Y receptor-evoked Ca^{2+} release and the response to trains of normally paced stimuli fused to generate prolonged $[Ca^{2+}]_i$ increases. Furthermore, elevation of the temperature to physiological levels (36°C) resulted in a more sustained depolarization-evoked Ca^{2+} increase compared with more transient or oscillatory responses at 20–24°C. The ability of signalling via a G-protein-coupled receptor to be potentiated by action potential waveforms and small amplitude depolarizations has broad implications in excitable and non-excitable tissues.

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G-protein-coupled receptors (GPCRs) are the largest family of cell surface receptors. They are responsible for transducing external stimuli into cellular activity and represent the principal targets for therapeutic intervention, particularly in the cardiovascular system (Rockman *et al.* 2002). A variety of evidence now supports the concept that a signalling via number of GPCRs can be controlled by changes in the cell membrane potential. For example, Ca^{2+} release stimulated by either muscarinic receptors in guinea-pig coronary artery smooth muscle or P2Y receptors in rat megakaryocytes is potentiated by depolarization and inhibited by hyperpolarization (Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999; Mason *et al.* 2000). The underlying mechanism is unknown, but can be explained by voltage control of IP_3 production, as suggested by Itoh *et al.* (1992) during activation of adrenergic receptors in rabbit mesenteric artery smooth muscle. An alternative explanation, as discussed in detail elsewhere (Mahaut-Smith *et al.* 1999; Mason & Mahaut-Smith, 2001), is that voltage-dependent configurational coupling between proteins in the surface and endoplasmic reticular membranes can modify IP_3-dependent Ca^{2+} release.

In the megakaryocyte and coronary or mesenteric artery smooth muscle, preactivation of a GPCR was required to observe voltage control of Ca^{2+} release or IP_3 generation (Itoh *et al.* 1992; Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999). However, there are also examples where constitutive voltage control of IP_3-dependent Ca^{2+} release has been described, including skeletal muscle (Vergara *et al.* 1985; Araya *et al.* 2003), smooth muscle (Best & Bolton, 1986; Suzuki & Hirst, 1999; Van Helden *et al.* 2000) and the giant algae *Chara* (Wacke & Thiel, 2001). GPCRs can generate responses in the absence of agonists (Seifert & Wenzel-Seifert, 2002), and therefore regulation of GPCR activity may also be responsible for the apparent intrinsic voltage control of IP_3-dependent Ca^{2+} release in these tissues. It is also worth noting that absorption of light

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by rhodopsin, the most widely studied GPCR, generates a charge displacement comparable with the gating currents of voltage-dependent ion channels (Cone, 1967; Sullivan & Shukla, 1999). Thus, transmembrane voltage may directly control GPCR activation and thereby regulate other downstream targets of this class of receptor such as adenylate cyclase and ion channels (Dascal, 2001). Indeed, the activation of G-protein-activated inwardly rectifying K^+ channels by M_2 muscarinic receptors expressed in *Xenopus* oocytes has recently been suggested to be voltage-dependent (Ben Chaim *et al.* 2003).

The extent to which Ca^{2+} signalling through GPCRs can be directly controlled by the membrane potential is unknown. Using the non-excitable rat megakaryocyte as a model system, we now show that voltage control of P2Y receptor-evoked Ca^{2+} release is graded, without evidence for a threshold potential, such that this signalling pathway can be controlled by small amplitude and short duration fluctuations of membrane voltage. The marked voltage sensitivity of signalling via this receptor allows cardiac action potential waveforms to significantly potentiate ADP-evoked Ca^{2+} mobilization. Therefore, the direct regulation of GPCR signalling by the cell potential should be more widely considered.

Methods

Cell isolation

Male adult (>150 g) Wistar rats were killed by exposure to a rising concentration of CO_2 followed by cervical dislocation, in accordance with UK Home Office guidelines. Marrow cells were isolated from femoral and tibial bones as previously described (Mahaut-Smith *et al.* 1999) in standard external saline (see below) containing 0.32 U ml^{-1} type VII apyrase (Sigma-Aldrich, Poole, UK). Apyrase was present during the preparation and storage of cells, but omitted during experiments. Megakaryocytes were distinguished on the basis of their large size and recordings were made 2–12 h after marrow removal.

Solutions

The standard external saline contained (mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose, titrated to pH 7.35 with NaOH. For Ca^{2+}-free saline, $CaCl_2$ was replaced by an equal concentration of $MgCl_2$, and where stated 0.5 mM Na_2EGTA also included. For Na^+-free saline, NaCl was replaced by *N*-methyl-D-glucamine (NMDG) and the pH adjusted to 7.35 with HCl. The pipette saline contained (mM): 150 KCl, 2 $MgCl_2$, 0.1 EGTA, 0.05 Na_2GTP, 0.05 K_5fura-2, 10 Hepes, adjusted to pH 7.2 with KOH. K_5fura-2 was purchased from Molecular Probes (Eugene, OR, USA). ADP (Sigma, UK) was treated by incubation with hexokinase and glucose to remove contaminating levels of ATP as previously described (Mahaut-Smith *et al.* 2000) and was applied to the cells via gravity-driven bath superfusion.

Electrophysiology

Conventional whole-cell patch clamp recordings were carried out in voltage clamp mode using an Axopatch 200B amplifier (Axon Instruments, CA, USA). pCLAMP and a Digidata interface (Axon Instruments) were used to deliver either voltage steps or action potential waveforms (APWs) derived from Oxsoft Heart 4.8 (Noble, 1999). To study the dependence of the P2Y receptor-evoked Ca^{2+} response on voltage pulse amplitude and duration, depolarizing and hyperpolarizing steps of increasing amplitude or duration were applied over two overlapping ranges. For increasing duration, the ranges were 25–300 ms in 25 ms increments and 100–1500 ms in 100 ms increments, with a holding potential of –85 mV and a depolarization of 135 mV. For increasing amplitude, the ranges were 1–5 mV in 1 mV increments and 5–75 mV in 5 mV increments, with a holding potential of –75 mV and a duration of 5 s. Due to desensitization of responses following large amplitude depolarizations, the maximum Ca^{2+} increase was also assessed in individual cells during the application of repeated steps of 40, 60 and 80 mV amplitudes from –75 mV. Action potentials were scaled to give a resting membrane potential of –85 mV with a maximum overshoot of +46 and +42 mV for ventricular and atrial APWs, respectively, as previously described (Lu *et al.* 2001). Recordings were made at room temperature (20–24°C), or at 36°C by regulation of the chamber temperature as previously described (Lu *et al.* 2001).

Fluorescence measurements

A Cairn spectrophotometer system (Cairn Research Ltd, Kent, UK) coupled to a Nikon Diaphot inverted microscope (Nikon, Japan) was used to measure fura-2 fluorescence as described in detail elsewhere (Mahaut-Smith, 1998). Fura-2 fluorescence signals were sampled at 60 Hz, averaged to give a final acquisition rate of 15 Hz and exported for analysis within Microcal Origin (Microcal Software Inc., Northampton, MA, USA). For presentation, some traces were filtered in Origin using 3–5 point averaging. Calibration constants R_{min} and R_{max} were

measured extracellularly and a calibration kit (Molecular Probes) was used to derive a K_d for fura-2 at room temperature (258 nM). For experiments at 36°C, the K_d was corrected as previously described (Shuttleworth & Thompson, 1991), yielding a value of 216 nM. After application of a viscosity correction factor (0.85) to R_{min} and R_{max} (Poenie, 1990), background-corrected 340 nm/380 nm fluorescence ratios were converted to $[Ca^{2+}]_i$ as previously described (Grynkiewicz *et al.* 1985; Mahaut-Smith *et al.* 1999). Data are expressed as the means ± S.E.M. with statistical difference assessed using Student's unpaired *t* test.

Results

Relationship between depolarization amplitude and $[Ca^{2+}]_i$ increase

In the non-excitable rat megakaryocyte, depolarization from a holding potential of –75 mV has no effect on $[Ca^{2+}]_i$ in unstimulated cells, yet can repeatedly generate single or multiple spikes of Ca^{2+} increase during exposure to the P2Y receptor agonist ADP (see Fig. 1*A* and Mahaut-Smith *et al.* 1999). Application of increasing amplitude depolarizing steps of 5 s duration showed that the peak $[Ca^{2+}]_i$ response is graded with pulse magnitude up to approximately 40 mV (Fig. 1*A* and *B*, circles). The response tended to desensitize during repeated large amplitude depolarizations (≥40 mV; not shown), which could partially account for the reduced response at larger potential steps in the series (Fig. 1*B*, circles). Therefore, we also measured the maximal $[Ca^{2+}]_i$ increase in individual cells during repeated application of either 40, 60 or 80 mV amplitude, 5 s duration, depolarizing steps from –75 mV, applied at the same frequency as in Fig. 1*A* (Fig. 1*B*, triangles). Although a large variation between cells was observed, these data suggest that the response increases with depolarization magnitude up to at least 80 mV. Previous studies have shown that this response to depolarization results predominantly from Ca^{2+} release, supplemented by concomitant store-dependent Ca^{2+} influx (Mahaut-Smith *et al.* 1999). Figure 1*C* shows an example of a response in Ca^{2+}-free medium, demonstrating that Ca^{2+} release could be repeatedly stimulated by a series of increasing amplitude depolarizations. However, run-down of store content in Ca^{2+}-free medium complicated the detailed study of the response under these conditions.

Depolarization potentiated the ADP-evoked Ca^{2+} response in all megakaryocytes (120/120 cells in this study), although the magnitude of the response displayed

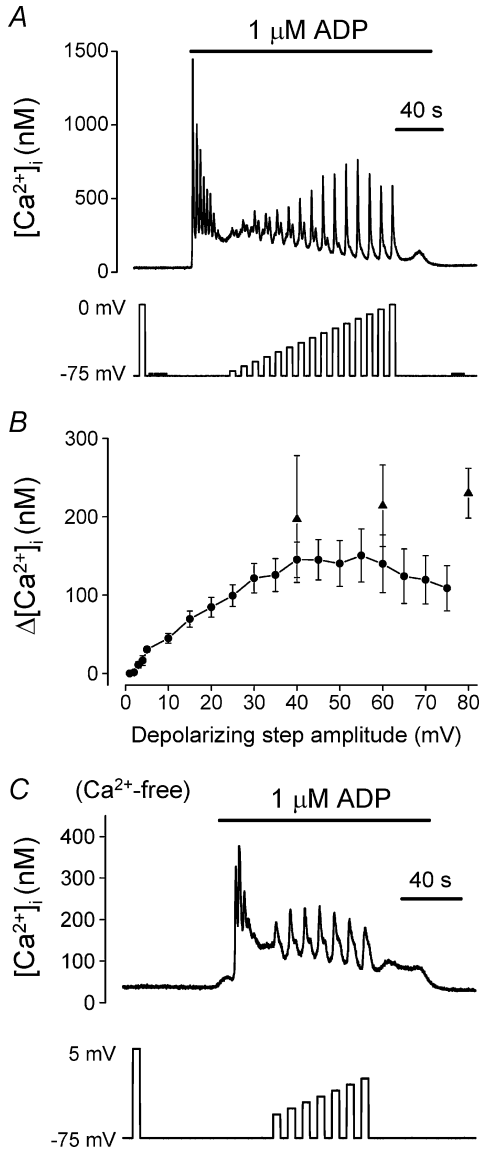

Figure 1. Effect of pulse amplitude on the depolarization-evoked $[Ca^{2+}]_i$ increase during stimulation of P2Y receptors

$[Ca^{2+}]_i$responses of rat megakaryocytes to ADP (1 μM, horizontal bar) and step depolarizations from a holding potential of –75 mV. The effect of depolarization during ADP application was assessed after the agonist-evoked increase had settled to a raised plateau level. *A*, effect of increasing the amplitude of the depolarizing step in 5 mV increments up to 75 mV. *B*, relationship between average peak $[Ca^{2+}]_i$ response and depolarizing pulse amplitude (mean ± S.E.M.). Data plotted as circles were from two series of increasing amplitude voltage steps; up to 75 mV in 5 mV increments (as shown in *A*; 18 cells) and up to 5 mV in 1 mV increments (9 cells). Data plotted as triangles represent the average (5–9 cells) maximal $[Ca^{2+}]_i$ increase during application of repeated 5 s duration pulses, of either 40, 60 or 80 mV amplitude, at the same frequency as in *A*. *C*, example of an experiment demonstrating that depolarization still potentiated ADP-evoked responses in a graded manner in Ca^{2+}-free saline.

marked single cell heterogeneity. A detectable $[Ca^{2+}]_i$ increase was observed in 100% of cells (27/27) following depolarizations of 5 mV, in 56% of cells (5/9) with steps of 3 mV, and in only a single cell (1/9; 11%) following a 2 mV step. This variability in absolute sensitivity most likely reflects the extent to which responses can be detected above the background noise as the cells with the largest Ca^{2+} increases were the ones that showed responses following depolarizations of only 2–3 mV. Overall, therefore, these data suggest that the phenomenon displays no discrete threshold for activation by depolarization.

Dependence on duration of the depolarizing step

The duration of a fixed-amplitude (–85 to +50 mV) depolarization was also a major factor in determining the magnitude of the Ca^{2+} increase during stimulation of P2Y receptors (Fig. 2*A*; data from 20 cells). The largest peak $[Ca^{2+}]_i$ increases were observed when the pulse length was $\geq\approx$500 ms, and gradually decreased as the duration was reduced. As described above for the sensitivity limit for pulse amplitude, the shortest duration required for a detectable response varied with the extent to which the cell displayed the voltage-dependent Ca^{2+} release phenomenon. $[Ca^{2+}]_i$ increases were detected following pulses as brief as 25 ms in the cells which displayed the most robust responses (5/11 cells, see for example Fig. 2*B*). Interestingly, the delay from depolarization to the first detectable Ca^{2+} increase did not vary with the duration of the step. The delay from the start of a 25 ms duration step was 440 ± 70 ms ($n = 5$), which was not significantly different ($P = 0.62$) from the delay when the pulse duration was 300 ms (480 ± 50 ms, $n = 7$). Thus, for the shorter pulses, a pronounced interval was observed between the end of a voltage step and the initial Ca^{2+} increase (Fig. 2*B*).

Relative role of hyperpolarization-induced inhibition of the P2Y receptor response

In both the megakaryocyte and coronary artery smooth muscle, hyperpolarizations are generally inhibitory to the agonist-evoked Ca^{2+} increases via P2Y and mACh receptors, respectively (Ganitkevich & Isenberg, 1993; Mason *et al.* 2000). We also tested the effect of varying the amplitude and duration of the hyperpolarizing step on the ADP-stimulated Ca^{2+} response once a raised plateau level had been achieved. The peak reduction in Ca^{2+} during a 10 s hyperpolarization from –45 mV was graded with the pulse amplitude (Fig. 3*A*) with no apparent threshold potential. The amplitude of the Ca^{2+} decrease to a fixed-amplitude hyperpolarization from –45 mV to –100 mV also increased in a graded manner with the pulse duration ($n = 19$; not shown). Although the effects of hyperpolarization on $[Ca^{2+}]_i$ during P2Y receptor activation were essentially the opposite of those of depolarization, the hyperpolarization-evoked decreases were always smaller in magnitude compared to the depolarization-evoked Ca^{2+} increases (see Fig. 3*A* and *B*). Consequently, in the experiment of Fig. 3*A*, the Ca^{2+} increases following repolarization to –45 mV were significantly larger than the hyperpolarization-induced decreases (Fig. 3*B*). Thus, although the voltage control of

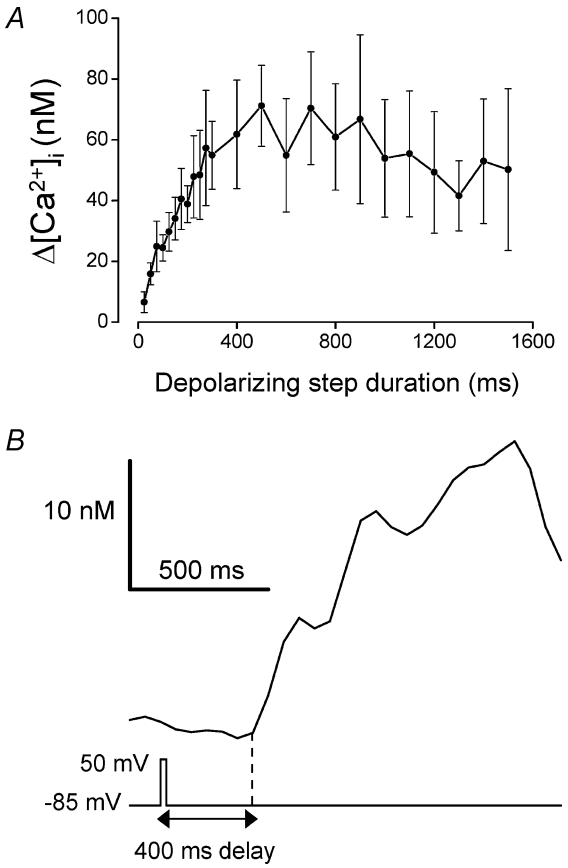

Figure 2. Effect of pulse length on the depolarization-evoked $[Ca^{2+}]_i$ increase during stimulation of P2Y receptors
Megakaryocytes were held at –85 mV and stepped to +50 mV for increasing durations (25–1500 ms) during the plateau phase of the 1 μM ADP-evoked Ca^{2+} response. *A*, relationship between average depolarization-evoked Ca^{2+} increase and pulse length. Data were obtained from two series of voltage protocols; 25–300 ms in 25 ms increments at a rate of 0.25 Hz (10 cells) and 100–1500 ms with 100 ms increments at a rate of 0.1 Hz (12 cells). *B*, $[Ca^{2+}]_i$ response to a 25 ms duration depolarization showing the characteristic long delay from the voltage step to initial Ca^{2+} increase. The delay from the start of the depolarization to the initial Ca^{2+} increase (vertical dashed line) was 400 ms.

ADP-evoked Ca^{2+} release in the megakaryocyte is bipolar in nature, the potentiation by depolarization dominates over hyperpolarization-mediated inhibition within the physiological range of membrane potentials.

Influence of single and multiple cardiac action potential waveforms on ADP-evoked Ca^{2+} responses

The concept that small or brief changes in the membrane potential can modify GPCR-induced Ca^{2+} signals has important implications for many cells, particularly in excitable tissues where larger changes of membrane potential regularly occur. Since the Ca^{2+} response increases with the period of depolarization up to about 500 ms, the phenomenon may have particular relevance in cardiac myocytes which display long duration action potentials and possess GPCRs and functional IP_3 receptors (Gorza

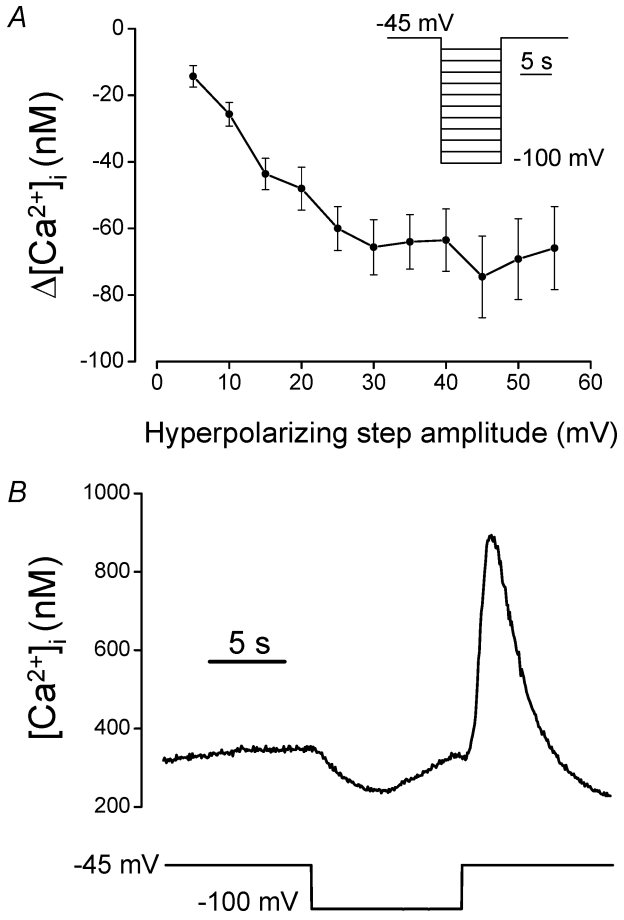

Figure 3. Hyperpolarization-evoked $[Ca^{2+}]_i$ decreases during exposure to ADP

Megakaryocytes were held at –45 mV and stepped to different hyperpolarized potentials for 10 s duration during the plateau phase of the ADP-evoked $[Ca^{2+}]_i$ increase. *A*, average peak change in $[Ca^{2+}]_i$ against amplitude of the hyperpolarizing voltage step. The values are means ± S.E.M. from 13 cells. Hyperpolarizations to potentials of –50 mV to –100 mV were applied in 5 mV increments (see inset) with an interpulse interval of either 10 or 20 s. *B*, $[Ca^{2+}]_i$ response to a 10 s duration voltage step to –100 mV, illustrating the typical larger magnitude of the depolarization-evoked Ca^{2+} increase upon repolarization to –45 mV compared with the initial hyperpolarization-induced Ca^{2+} decrease.

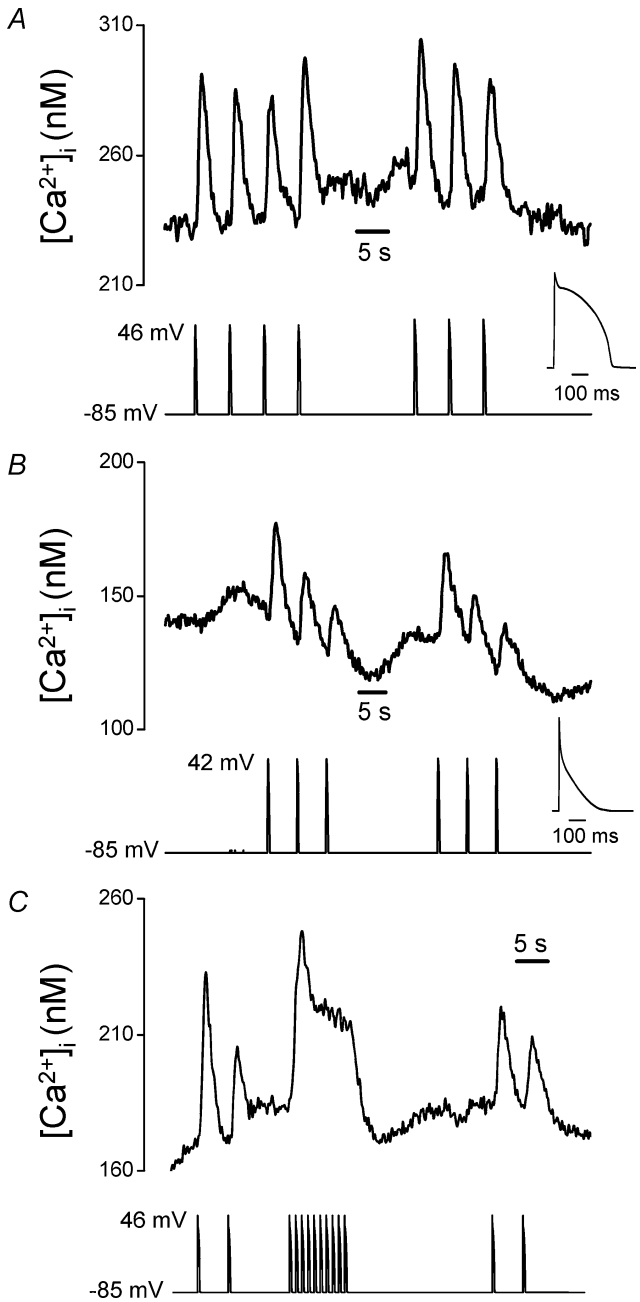

Figure 4. Cardiac action potential waveforms (APWs) repeatedly stimulate Ca^{2+} mobilization by potentiation of P2Y receptor signalling

$[Ca^{2+}]_i$responses in the presence of 1 μM ADP to repeated ventricular (*A*) and atrial (*B*) APWs applied at 0.2 Hz. The insets show the shape of the normally paced waveforms on an expanded timescale. *C*, $[Ca^{2+}]_i$ responses to ventricular APWs, applied at both 0.2 Hz and 1 Hz.

et al. 1993; Lipp *et al.* 2000; Rockman *et al.* 2002). Ion channels can have markedly different responses to APWs compared with simple step depolarizations (McCobb & Beam, 1991; Lu *et al.* 2001), therefore we examined the effect of APWs on $[Ca^{2+}]_i$ during P2Y receptor activation in the megakaryocyte. Individual ventricular (Fig. 4*A*) and atrial (Fig. 4*B*) action potential waveforms repeatedly elevated $[Ca^{2+}]_i$ during exposure to 1 μM ADP. Neither waveform had an effect on $[Ca^{2+}]_i$ under resting conditions (not shown, $n = 23$). The peak Ca^{2+} increase in response to a single action potential varied between cells, as predicted from the marked heterogeneity of the response, and was 159 ± 30 nM ($n = 13$) for ventricular and 47 ± 11 nM ($n = 10$) for atrial APWs. This Ca^{2+}-mobilizing effect of action potential waveforms was due primarily to the modulation of Ca^{2+} release, since it was present in Ca^{2+}-free, 0.5 mM EGTA ($n = 6$, not shown) and Na^+-free ($n = 4$, not shown) saline solutions, as previously demonstrated for the response to step depolarizations (Mahaut-Smith *et al.* 1999). The APWs used in the experiment of Fig. 4*A* and *B* were from normally paced hearts, but applied at a frequency of 0.2 Hz, therefore we also examined the effect of ventricular APWs at their normal frequency of 1 Hz. At this faster rate, the responses to individual action potentials fused to generate an elevated plateau level of Ca^{2+} (Fig. 4*C*, $n = 10$).

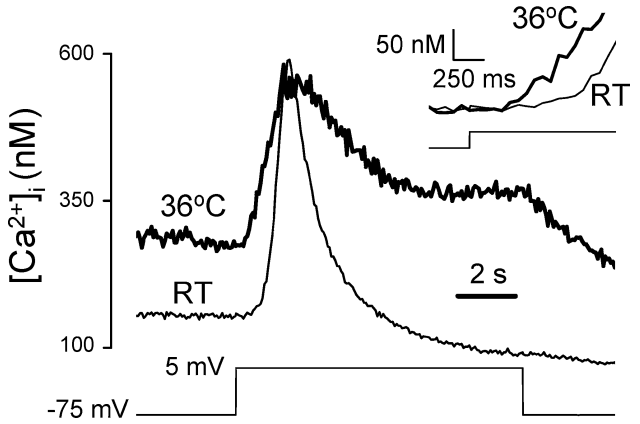

Figure 5. Comparison of depolarization-evoked responses at room temperature and physiological temperatures
$[Ca^{2+}]_i$ response to a depolarizing voltage step of 80 mV and 10 s duration in the presence of 1 μM ADP at room temperature (RT) and 36°C. Data are from two different megakaryocytes, representative of a total of 11 cells at 36°C and 8 cells at room temperature (RT, 20–24°C). The inset shows the onset of the responses on a faster timescale. To allow comparison of the kinetics at the two temperatures, the Ca^{2+} recording at 36°C was offset to start from the same level as at RT.

Effect of an elevation in temperature to physiological levels

Since all experiments to date on the voltage control of megakaryocyte P2Y-mediated Ca^{2+} signals have been conducted at room temperature (RT; 20–24°C) (Mahaut-Smith *et al.* 1999; Mason *et al.* 2000; Mason & Mahaut-Smith, 2001; Thomas *et al.* 2001), we also examined the response at physiological temperatures. In the presence of 1 μM ADP, typical Ca^{2+} increases at RT and 36°C in response to 80 mV, 10 s steps from a holding potential of –75 mV are shown in Fig. 5. The higher temperature resulted in a raised plateau level during ADP stimulation and had two main effects on the depolarization-evoked Ca^{2+} increase. Firstly, the response was converted from a transient to a more sustained response, with a significant increase in the integral above the plateau level (36°C *versus* RT: 1047 ± 178 *versus* 586 ± 105 nM s, respectively, $P < 0.05$, $n = 11$), despite a reduction of the peak increase (36°C *versus* RT: 204 ± 62 *versus* 316 ± 31 nM, respectively, $P < 0.01$, $n = 11$). Secondly, the time delay from depolarization to the first detectable increase was reduced (36°C *versus* RT: 0.3 ± 0.04 *versus* 0.6 ± 0.06 s, respectively, $P < 0.01$, $n = 11$) (see Fig. 5 inset, in which the predepolarization $[Ca^{2+}]_i$ values have been normalized for the two temperatures). Thus, increasing the experimental temperature to physiological levels accelerates the response to voltage and prolongs the Ca^{2+}-mobilizing effects of depolarization during ADP stimulation.

Discussion

In this study we address the extent to which signalling via GPCRs, a major class of transmembrane proteins not normally considered to be voltage dependent, can be controlled by changes in the cell membrane potential. We selected P2Y receptors in the rat megakaryocyte as a model system due to the absence of ryanodine receptors and voltage-gated Ca^{2+} and Na^+ channels in this typical non-excitable cell (Uneyama *et al.* 1993; Somasundaram & Mahaut-Smith, 1994; Mahaut-Smith *et al.* 1999; Mason & Mahaut-Smith, 2001). In addition, P2Y receptors are expressed in a range of tissues, including the cardiovascular and nervous systems (Kunapuli & Daniel, 1998; Moore *et al.* 2000), and so understanding the regulation of P2Y receptors has broad biomedical implications.

Previous work has shown that depolarization amplifies, whereas hyperpolarization inhibits, ADP-evoked release of Ca^{2+} from IP_3-dependent intracellular stores in the megakaryocyte (Mahaut-Smith *et al.* 1999; Mason *et al.* 2000; Mason & Mahaut-Smith, 2001). We now show that

the voltage control of P2Y receptor-evoked Ca^{2+} release in the megakaryocyte displays no threshold potential, such that depolarizations of only a few millivolts can potentiate the ADP-evoked Ca^{2+} increase. The phenomenon was also sufficiently sensitive for depolarizing steps of only 25 ms duration over the voltage range observed for action potentials (–85 to +50 mV) to generate significant Ca^{2+} increases. Hyperpolarizations inhibited the ADP-evoked Ca^{2+} increase in a graded manner, also without evidence for a threshold voltage or minimum duration. This further indicates that the underlying mechanism is bipolar in nature. However, regardless of the pulse length or amplitude, depolarization-mediated increases always dominated over the hyperpolarization-induced inhibition for square wave pulses. It was also interesting that during a 5 s duration depolarization or hyperpolarization at room temperature, the evoked $[Ca^{2+}]_i$ change was transient and eventually came to rest at a level similar to the prestimulus plateau (although altered driving forces for Ca^{2+} entry most likely account for shifts in the steady state levels particularly at room temperature; see for example Figs 3*B* and 5). This suggests that the mechanism predominantly responds to changes in potential rather than steady state transmembrane potential levels. From these properties, it would be predicted that repeated cardiac action potentials would potentiate Ca^{2+} release during P2Y stimulation and indeed this was observed for both atrial and ventricular waveforms (see Fig. 4*B*). It is unclear at present why the response to depolarization is more sustained at 36°C compared to room temperature (Fig. 5); however, this would tend to enhance the potentiation of ADP-evoked responses by action potentials at physiological temperatures. Interestingly, due to the irreducible delay in the depolarization-evoked Ca^{2+} response described above, the Ca^{2+} increase commenced after completion of the atrial APW and towards the end of the ventricular APW. Together with the fact that the underlying mechanism appears to respond to changes in potential rather than steady state levels (see above), this resulted in a prolonged plateau of Ca^{2+} increase following multiple APWs at their normal frequency of 1 Hz (Noble, 1984). GPCRs, including P2Y purinoceptors, and functional IP_3 receptors (IP_3Rs), are present in the heart where this mechanism could have important physiological consequences (Lipp *et al.* 2000; Vassort, 2003). Furthermore, during pathophysiological states such as heart failure, the levels of IP_3R expression are known to increase (Go *et al.* 1995) and ventricular APWs are prolonged (Tomaselli *et al.* 1994). For neuronal tissue, this could represent an important means whereby changes in membrane potential, can modify postsynaptic signalling via GPCRs (see Dascal, 2001, for review). In non-excitable tissues, significant fluctuations in membrane potential are often associated with agonist stimulation and could alter the resultant Ca^{2+} signals via the mechanism we describe here. Indeed we have previously shown that the shifts in membrane potential associated with repeated activation of Ca^{2+}-dependent K^+ channels can induce small $[Ca^{2+}]_i$ oscillations due to repeated release from intracellular stores in the rat megakaryocyte (Mason *et al.* 2000).

The megakaryocyte is frequently used as a model for signalling in its product, the anuclear platelet. An increasing number of studies now suggest that most platelet receptors are expressed on the surface of the mature megakaryocyte and signal via identical pathways (Ikeda *et al.* 1992; Briddon *et al.* 1999; Vial *et al.* 2002). Platelets possess three P2 receptors; one ionotropic ($P2X_1$) and two GPCRs ($P2Y_1$ and $P2Y_{12}$) (Gachet, 2001; Kunapuli *et al.* 2003). In the present study, we selectively activated P2Y over $P2X_1$ receptors using hexokinase-purified ADP (Mahaut-Smith *et al.* 2000). $P2Y_1$ receptors signal primarily via $G\alpha_q$ and phospholipase-Cβ to release Ca^{2+} from internal stores, whereas $P2Y_{12}$ receptors are coupled to $G\alpha_i$, resulting in an inhibition of adenylate cyclase and activation of PI3-kinase (Gachet, 2001; Kunapuli *et al.* 2003). $P2Y_1$ and $P2Y_{12}$ receptor-specific knock-outs have confirmed that the ADP-evoked Ca^{2+} response in the platelet is mediated primarily via $P2Y_1$ receptors (Leon *et al.* 1999; Fabre *et al.* 1999; Foster *et al.* 2001). Thus, $P2Y_1$ receptors coupled to $G\alpha_q$ and phospholipase-Cβ most likely represent the ADP receptor pathway controlled by membrane potential in the megakaryocyte.

To date, Ca^{2+} signalling via four different GPCRs has been suggested to be directly controlled by the membrane potential: mACh receptors in coronary artery smooth muscle and heterologously expressed in *Xenopus* oocytes, adrenergic receptors in mesenteric artery smooth muscle, and $P2Y_1$ and thromboxane A_2 receptors in rat megakaryocytes (Itoh *et al.* 1992; Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999; Mason & Mahaut-Smith, 2001; Ben Chaim *et al.* 2003). In each case, preactivation of the receptor was required to detect voltage modulation of intracellular Ca^{2+} release (or in the case of adrenergic receptors, IP_3 generation). All four receptors are members of the rhodopsin/β2 adrenergic-like family (family A) of GPCRs (Gether, 2000). Therefore, it is particularly interesting that the activation of rhodopsin generates a conformation-dependent charge movement similar to the gating currents of voltage-dependent ion channels (Cone, 1967; Sullivan & Shukla, 1999). Any event that generates movement of charges within the

membrane would be expected to be sensitive to the transmembrane potential and therefore the activation of rhodopsin and related GPCRs could be a voltage-dependent process. Indeed, Ben Chaim *et al.* (2003) have recently proposed that M_2 muscarinic receptors expressed in *Xenopus* oocytes are voltage dependent and that the voltage sensor lies in the region of the receptor which interacts with the heterotrimeric G-protein. Another possibility to explain the voltage control of P2Y receptors and other GPCRs is that ligand binding within its active site is influenced by changes in the transmembrane potential. Activation of NMDA receptors by depolarization is a well-established example whereby modulation of binding by an external ion (in this case Mg^{2+}) accounts for the voltage sensitivity of a transmembrane receptor. Site-directed mutagenesis studies of ligand-binding sites within the $P2Y_1$ receptor indicate that two out of the three amino acids with the most significant effect on receptor activation are positively charged arginines (Jiang *et al.* 1997). These presumably reflect binding to the negatively charged phosphate moieties of ADP and thus represent potential interactions that could be modified by the transmembrane voltage. Although ligand–receptor interaction or conformational state of the receptor are the main candidates for the voltage-dependent processes during P2Y receptor signalling, the activity of either the heterotrimeric G-protein or phospholipase-Cβ cannot at present be excluded. For example in neurones, depolarization modulates G-protein inhibition of Ca^{2+} channels by displacing $\beta\gamma$ subunits from their target (Golard & Siegelbaum, 1993; Zamponi & Snutch, 1998). However, compared to the voltage-dependent Ca^{2+} release phenomenon in the megakaryocyte, this effect requires larger amplitude depolarizations and reduces rather than potentiates the action of the GPCR on its target. A further mechanism that can explain the phenomenon in the megakaryocyte is one of configurational coupling, as proposed between transient receptor potential (TRP) channels and IP_3 receptors on the endoplasmic reticulum (Kiselyov *et al.* 1998). However, one would expect this to involve very little delay and we observe a delay of ≥ 400 ms between depolarization and the initial Ca^{2+} increase in the megakaryocyte (Fig. 2*B*). This delay is comparable with the time required for ADP-dependent generation of IP_3 and Ca^{2+} release in the megakaryocyte (Somasundaram & Mahaut-Smith, 1994; Vial *et al.* 2002). Together with the reduced delay at higher temperatures (Fig. 5), these data indicate the involvement of a metabolic step rather than one that depends purely upon a configurational coupling between plasma and internal store proteins. The modulation of $P2Y_1$ receptor-evoked IP_3 production by the membrane potential, as opposed to configurational coupling, is further supported by the indistinguishable characteristics of ADP- and depolarization-evoked waves in the megakaryocyte (Thomas *et al.* 2001). In summary, most evidence points towards voltage control of P2Y receptor binding or its conformation, leading to altered IP_3 production and subsequent Ca^{2+} release. Production of $IP_3$by phospholipase-Cβ is just one example of a vast number of different signalling events that are controlled by GPCRs. Other common GPCR-regulated pathways include adenylate cyclase, and thus cAMP production, and direct effects on ion channels (Dascal, 2001). Such signalling cascades may also be under the control of membrane voltage, as described for Ca^{2+} release in our studies.

A further key question is why P2Y receptor-evoked Ca^{2+} responses in the megakaryocyte are so markedly potentiated by depolarization and inhibited by hyperpolarization. In some non-excitable cell types, such as mast cells and lymphocytes (Penner *et al.* 1988; Lewis & Cahalan, 1989), the main influence of membrane potential during agonist stimulation results from alterations of driving force on Ca^{2+} influx and therefore the effects on $[Ca^{2+}]_i$ are the opposite of those observed in the megakaryocyte. Thus, the voltage-dependent mechanism may not be present, or as dominant, during agonist-evoked Ca^{2+} signalling in these other cells. In coronary artery smooth muscle, where depolarization potentiates Ca^{2+} release during stimulation of mACh receptors (Ganitkevich & Isenberg, 1993), larger amplitude voltage steps are required compared to during activation of P2Y receptors in the megakaryocyte. This may again be due to a difference in receptor type or ligand–receptor interaction. Another possibility is that the membrane invagination system of the megakaryocyte, which functions to provide additional membrane for platelet formation, amplifies an innate voltage dependence to P2Y receptor signals. Recent specific capacitance measurements have shown that these invaginations increase the amount of surface-connected membrane by 4- to 14-fold (Mahaut-Smith *et al.* 2003). This is equivalent to the specific capacitance of mammalian skeletal muscle, where the t-tubular system increases the amount of surface-connected membrane. Membrane invaginations may enhance voltage-dependent Ca^{2+} release via P2Y receptors by increasing the ratio of surface membrane (and thus receptor number) to cytoplasmic volume. In this scenario, the phenomenon may be more important in cells with a high specific capacitance such as skeletal muscle and certain types of cardiac muscle. In addition, cell compartmentalization can increase the ratio of surface membrane to cytoplasmic

volume, for example in dendritic spines. These foci of neuronal integration express functional IP_3 receptors (Miyata *et al.* 2000) and represent one structure where modulation of GPCRs by ionotropic receptors or action potentials would have clear physiological relevance.

In conclusion, we have demonstrated that Ca^{2+} signals evoked via $P2Y_1$ receptors can be altered by changes in membrane potential of only a few millivolts or of only short duration, including action potential waveforms. This marked sensitivity of a GPCR of the rhodopsin family to transmembrane voltage has important implications for the regulation of GPCR signalling in all cell types.

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