Direct Voltage Control of Signaling via P2Y₁ and Other Gαq-coupled Receptors*

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Emerging evidence suggests that Ca²⁺ release evoked by certain G-protein-coupled receptors can be voltage-dependent; however, the relative contribution of different components of the signaling cascade to this response remains unclear. Using the electrically inexcitable megakaryocyte as a model system, we demonstrate that inositol 1,4,5-trisphosphate-dependent Ca²⁺ mobilization stimulated by several agonists acting via Gαq-coupled receptors is potentiated by depolarization and that this effect is most pronounced for ADP. Voltage-dependent Ca²⁺ release was not induced by direct elevation of inositol 1,4,5-trisphosphate, by agents mimicking diacylglycerol actions, or by activation of phospholipase Cγ-coupled receptors. The response to voltage did not require voltage-gated Ca²⁺ channels as it persisted in the presence of nifedipine and was only weakly affected by the holding potential. Strong predepolarizations failed to affect the voltage-dependent Ca²⁺ increase; thus, an alteration of G-protein βγ subunit binding is also not involved. Megakaryocytes from P2Y₁−/− mice lacked voltage-dependent Ca²⁺ release during the application of ADP but retained this response after stimulation of other Gαq-coupled receptors. Although depolarization enhanced Ca²⁺ mobilization resulting from GTPγS dialysis and to a lesser extent during AlF₄⁻ or thimerosal, these effects all required the presence of P2Y₁ receptors. Taken together, the voltage dependence to Ca²⁺ release via Gαq-coupled receptors is not due to control of G-proteins or downstream signals but, rather, can be explained by a voltage sensitivity at the level of the receptor itself. This effect, which is particularly robust for P2Y₁ receptors, has widespread implications for cell signaling.

G-protein-coupled receptors (GPCRs) constitute the largest family of surface proteins and represent key targets for therapeutic intervention (1, 2). Although GPCR activation is not normally considered to be directly sensitive to changes in the cell membrane potential, studies in a variety of cell types now support this concept (3–7). In particular, Ca²⁺ release stimulated by P2Y receptors in rat megakaryocytes or muscarinic cholinergic receptors in coronary artery smooth muscle is potentiated by depolarization and inhibited by hyperpolarization (5, 7, 8). The underlying mechanism is unknown and may lie at the level of the receptor, a downstream signaling molecule, or reflect a direct effect on the intracellular Ca²⁺ stores.

Voltage control of GPCR signaling represents a potentially important means whereby electrogentic influences can modify cellular signaling. Indeed, a range of physiological voltage waveforms from slow oscillations to action potentials can alter the Ca²⁺ mobilization induced by P2Y receptor activation (8, 9). Constitutive voltage control of phospholipase C and, thus, IP₃-dependent Ca²⁺ release has also been described in smooth and skeletal muscle (10, 11), inferring that activation of heterotrimeric G-proteins or their receptors can be voltage-dependent in the absence of exogenous agonist. To date, the voltage dependence to GPCR signaling has been most extensively studied in the rat megakaryocyte, where the lack of voltage-gated Ca²⁺ influx and ryanodine receptors simplifies studies of IP₃-dependent Ca²⁺ mobilization (12–14). In addition, due to its role in generating functional anucleated blood platelets, the megakaryocyte cell surface possesses a plethora of platelet surface receptors with relatively well defined signaling pathways (15). We have now used the megakaryocyte, including tissues from receptor-deficient mice, to investigate the location of the voltage sensor within the signaling cascade coupled to P2Y and other Gαq-coupled receptors.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation and Animals**—Marrow was dissociated from the femoral and tibial bones of male adult C57BL/6 mice and Wistar rats into standard external saline (see “Solutions and Reagents”) containing 0.32 units/ml type V or type VII apyrase. Apyrase was present throughout storage but omitted during experiments except where indicated. Rat marrow provides a higher yield of megakaryocytes compared with the mouse and, thus, was used for many experiments due to the rarity (<1%) of these cells. Megakaryocytes were distinguished on the basis of their large size (16), and recordings were made within 12 h of marrow removal. P2Y₁ receptor-deficient (P2Y₁−/−) mice were generated as previously described (17).

**Solutions and Reagents**—The standard external saline contained 145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 10 mM D-glucose titrated to pH 7.35 with NaOH. Application of extracellular solutions was by gravity-driven bath superfusion. For Ca²⁺-free saline, CaCl₂ was replaced by an equal concentration of MgCl₂. For Na⁺-free saline, Na⁺ was replaced by an equal concentration of n-methyl-D-glucamine, and the pH was titrated to 7.35 with n-methyl-D-glucamine. The pipette saline contained 150 mM KCl, 2 mM MgCl₂, 0.1 mM EGTA, 0.05 mM Na₂GTP, 10 mM Hepes, pH 7.2 (adjusted with KOH), and either 0.05 mM K₂fura-2 or 0.05 mM (NH₄)₂fluor-3. For experiments with
internal GTP·S (tetrалiθium salt; Sigma-Aldrich), Na5GTP was omitted from the pipette saline. Kfura-2 and (NH4)2-3fluor were purchased from Molecular Probes (Eugene, OR). ADP (Sigma) was pretreated for 1 h with hexokinase and high glucose to remove contaminating levels of ATP, which co-activates P2X receptors, as described previously (18). Caged-IP3, caged-G-PIP2, and 1-oleoyl-2-acetylglceryl (OAG) were obtained from (Calbiochem-Novabiochem). Platelet-derived growth factor-BB (PDGF) and phorbol 12-myristate 13-acetate were purchased from Sigma. Cells were exposed to AIF2 by the addition of 5 mM NaF and 100 mM AlCl3 to the normal external saline. For assessment of the relative dependence to different receptors, we selected agonist concentrations that generated a robust Ca2+ response and which were greater than the reported EC50 value in the plateau and/or megakaryocyte (19–22). This will limit concentration-dependent effects for the depolarization-evoked increase, which varies by only 0.74-fold over a 100-fold-concentration range above the EC50 value.2

Electrophysiology—Conventional whole-cell patch clamp recordings were carried out in voltage clamp mode using an Axopatch 200B amplifier (Axon Instruments, CA) with 70–75% series resistance compensation. pCLAMP and a Digidata interface (Axon instruments) were used to deliver voltage steps. Voltage-dependent Ca2+ mobilization was assessed using 80-mV depolarizations of 2–10-s duration, which stimulate a near-maximal voltage-dependent [Ca2+]i increase during exposure to the agonist ADP (9). For the experiments shown in Fig. 3C, the specified holding potential was applied before ADP, and the effect of the 80-mV depolarization was assessed as soon as possible after the initial agonist-evoked [Ca2+]i transients. Although this approach may have introduced some variation in internal Ca2+ store content as a consequence of different driving forces for Ca2+ influx, it avoided the voltage-dependent Ca2+ responses caused by changing the holding potential during activation of the G-protein-coupled receptor. Recordings were made at room temperature (20–24°C) for improved stability of recordings, although we have previously confirmed that voltage control of P2Y receptor-evoked Ca2+ release also occurs at normal body temperatures (9).

Fluorescence Measurements—Fura-2 or fluo-3 fluorescence measurements of intracellular Ca2+ were made using a Cairn Spectrophotom-eter System (Cairn Research Ltd, Kent, UK) coupled to a Nikon Diaphot inverted microscope (Nikon, Japan), as described in detail elsewhere (23, 24). Excitation wavelengths, provided by a monochromator or using interference filters, were 340 and 380 nm for fura-2 and 490 nm for fluo-3 with emission bandwidths of ~480–600 and 528–600 nm, respectively. Fluorescence emission and electrophysiologcal data were simultaneously recorded to computer using Cairn acquisition hardware and software. Fluorescence data were sampled at 60 Hz, averaged to give a final acquisition rate of 15 Hz, and exported for analysis with Microcal Origin (Microcal Software Inc., Northampton, MA). For calibration of fura-2, the maximum and minimum 340/380-nm fluorescence ratios were measured extracellularly, and background-corrected ratios were converted to [Ca2+]i, as described previously (25) after application of a viscosity correction factor of 0.85 (23, 26). A Kd of 258 μM was derived using a calibration kit from Molecular Probes. Data are expressed as the means ± S.E. with the statistical difference assessed using Student’s unpaired t test. Fluo-3 fluorescent signals were background-subtracted, and f/f0 ratios were used to normalize fluorescence levels (f) against starting fluorescence (f0). Data are expressed as the means ± S.E. of the mean, where n is the number of cells. Statistical comparisons were made using Student’s unpaired t test, and p < 0.05 was considered significant. Flash Photolysis of Caged Compounds—Cells were loaded with caged-IP3 (100 μM) or caged-G-PIP2 (200 μM) by dialysis from the patch pipette. Uncaging was achieved using a Cairn Flash Photolysis unit (Cairn Research Ltd, Faversham, Kent, UK) with the amount of photolysis determined by the charge delivered to a high intensity xenon arc lamp (Advance Radiation, Inc., Santa Clara, CA). A 503-nm dichroic mirror with extended UV reflectance allowed simultaneous delivery of uncaging and 490-nm illumination as described in detail elsewhere (12, 23).

RESULTS

Voltage-dependent Ca2+ Release during Activation of Multiple Gαq-coupledGPCRs—The megakaryocyte is a useful cell type for studying voltage control of GPCR-evoked Ca2+ signaling as this electrically excitable cell type expresses multiple surface receptors known to couple to Ca2+ mobilization in the platelet. Consequently, the platelet agonists ADP, U46619 (a stable thromboxane A2 analogue), and 5-HT, which stimulate phospholipase Cβ via Gαq-coupled receptors (27–30), all evoked a robust elevation of intracellular Ca2+ concentration ([Ca2+]i) in the rat megakaryocyte (Fig. 1, A–C). In the absence of exogenous agonist, depolarization had little or no effect on [Ca2+]i (Fig. 1, A–E). However, despite the lack of functional voltage-gated Ca2+ channels (7, 13, 31), depolarization from −75 to +5 mV repeatedly stimulated a significant [Ca2+]i increase during stimulation by each of the three agonists tested.

The agonist-induced depolarization-evoked Ca2+ increase results primarily from release of intracellularly stored Ca2+, as it was of similar amplitude in Ca2+-free medium (see Fig. 1D) for a typical experiment with ADP; data are not shown for thromboxane A2 (n = 9) and 5-HT (n = 5). However, depletion of stores frequently led to a run-down of the response; therefore, subsequent experiments were conducted in the presence of external Ca2+. Furthermore, although voltage changes are capable of modulating the initial agonist-evoked Ca2+ signals, including oscillations (8, 32), depolarizations in this study were applied during the steady plateau phase of the response to clearly distinguish voltage-dependent effects. Fig. 1E compares the peak agonist and depolarization-evoked Ca2+ increases in the presence of external Ca2+. The rank order of ability for depolarization to potentiate Ca2+ mobilization during agonist stimulation was ADP > U46619 > 5-HT, measured both as the absolute voltage-dependent Ca2+ increase (315 ± 24, 118 ± 17, and 52 ± 14 nm for ADP (n = 19), U46619 (n = 18), and 5-HT (n = 13), respectively) and the percentage Ca2+ increase for depolarization compared with that evoked by the agonist (52 ± 5, 33 ± 6, and 11 ± 4% for ADP, U46619, and 5-HT, respectively). Thus, several Gαq-coupled receptors are able to induce the ability of membrane potential to control Ca2+ mobilization, although this response is most pronounced for ADP-stimulated P2Y receptors.

Lack of Voltage-dependent Ca2+ Release after Direct Elevation of IP3 and/or Diacylglycerol Analogues and Phospholipase CγActivation—The voltage dependence of Ca2+ release during activation of a GPCR can be explained by direct regulation of the receptor, its G-protein, or phospholipase C by the cell membrane potential (5, 7, 12, 33). To assess whether the products of phospholipase C can alone induce a voltage dependence to Ca2+ mobilization, we asked whether the agonist-induced depolarization-evoked Ca2+ release, for example by configurational coupling between IP3 receptors on the internal stores and ion channels on the plasma membrane (34), we tested the effects of elevating IP3 with and without stimulation of diacylglycerol (DAG)-dependent pathways. To limit the effect of reduced Ca2+ influx when the cell is depolarized, the duration of the voltage step was shortened to 2s, which we have recently shown maximally activates the voltage-dependent Ca2+ release process (9). Depolarizing voltage steps could not stimulate a Ca2+ increase after uncaging of IP3 by flash photolysis (n = 9; Fig. 2A). The lack of response to voltage was not due to complete inactivation of IP3 receptors or rapid degradation of IP3 since depolarization also failed to stimulate Ca2+ increase after photolysis of the poorly metabolizable but fully active IP3 analogue, G-PIP2, which itself could repeatedly release Ca2+ (n = 10; Fig. 2B) (35, 36). We also tested the effects of depolarization during application of the cell-permeant DAG analogue, OAG (20 μM, n = 5; 100 μM, n = 9). The Ca2+ response to this compound was variable at either concentration, ranging from no increase (3/14 cells) to a small (~150 nm) increase (11/14 cells), which may in part be due to stimulation of Ca2+ entry via TRPC channels (canonical members of the transient receptor potential family of ion channels) (37, 38). However, in all cells, depolarization of the membrane (from −75 to +5 mV, 10 s duration) failed to produce a Ca2+...
We also activated the DAG target protein kinase-C using phorbol 12-myristate 13-acetate at 200 nM (\(n=6\)) or 1 \(\mu\)M (\(n=10\)). As with OAG, phorbol 12-myristate 13-acetate evoked a Ca\(^{2+}\) increase in a proportion of cells (8/16); however, in all cells (\(n=16\)), depolarization from -75 to +5 mV failed to stimulate a Ca\(^{2+}\) increase (not shown). Finally, elevation of both IP\(_3\), by flash photolysis combined with either 1 \(\mu\)M phorbol 12-myristate 13-acetate (\(n=4\), not shown) or 100 \(\mu\)M OAG (\(n=8\); Fig. 2C) failed to induce voltage-dependent Ca\(^{2+}\) release. Together, these experiments exclude the possibility that the voltage dependence during GPCR activation results from an event induced by direct activation of IP\(_3\) receptors or DAG-dependent pathways.

Platelets and megakaryocytes also express receptors coupled to Ca\(^{2+}\) mobilization via phospholipase C\(_\gamma\), including those for platelet-derived growth factor (PDGF) (39). This agonist was slow to generate a Ca\(^{2+}\) increase, as expected at room temperature for this type of tyrosine-kinase-linked pathway, however no depolarization-induced response was observed leading up to or during the initial PDGF-induced Ca\(^{2+}\) increase (\(n=5\), Fig. 1).
Dihydropyridine Receptors and Other Voltage-gated Ca\textsuperscript{2+} Channels Are Not Involved in the Voltage Dependence to G\textsubscript{q} Protein-coupled Receptors—Depolarization-evoked IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release has been reported in skeletal and vascular smooth muscle, where dihydropyridine receptors coupled either directly or indirectly to G-proteins act as the voltage sensors (10, 11). Exogenous agonist is not required for the response; however, constitutive GPCR activity may still be involved. To test whether modified voltage-dependent Ca\textsuperscript{2+} channels are involved in the megakaryocyte voltage-gated Ca\textsuperscript{2+} release phenomenon, we examined the effects of a dihydropyridine and different holding potentials. Nifedipine (up to 50 \textmu M), a blocker of L-type Ca\textsuperscript{2+} channels and myocyte voltage-gated Ca\textsuperscript{2+} release, had no significant effect on the ability of depolarization to potentiate P2Y\textsubscript{1} receptors during activation by 1 \textmu M ADP in the rat megakaryocyte (Fig. 3, A and C). A high concentration of nifedipine was used because it inhibited the voltage-gated K\textsuperscript{+} currents, thereby acting as a positive control (see Fig. 3A). Neither short term (2–5 min) nor long term (>20 min) exposures to nifedipine significantly affected the peak of the voltage-dependent Ca\textsuperscript{2+} increase (Fig. 3B).

ADP-induced Ca\textsuperscript{2+} mobilization was potentiated by a depolarizing step (80 mV, 10-s duration) applied from a wide range of holding potentials, although the peak response gradually declined as the holding potential was shifted from −102.5 to +7.5 mV (Fig. 3C). This may in part result from the reduced driving force for Ca\textsuperscript{2+} entry and, thus, a lower level of Ca\textsuperscript{2+} in the stores as the cell is held more depolarized (see “Experimental Procedures”). However, it does demonstrate the ability of membrane voltage to release a significant amount of Ca\textsuperscript{2+} from internal stores at holding potentials of −20 mV and more depolarized. This further argues against the role of voltage-gated Ca\textsuperscript{2+} ion channels in the underlying mechanism, since these are largely inactive over this range of holding potentials (40). The series of experiments in Fig. 3C also demonstrates that the response to voltage is highly robust over the range of normal resting potentials, which fits with postulated roles for this phenomenon, namely control of agonist-evoked Ca\textsuperscript{2+} oscillations and synergy between post-synaptic potentials or action potentials and GPCRs (8, 9).

One well established, weakly voltage-dependent process during GPCR activation is the inhibitory action of G-protein \beta\gamma subunits on voltage-gated Ca\textsuperscript{2+} channels (41–43). We, therefore, tested a predepolarization protocol (175mV, 15-ms depolarization from −75 mV) that mimics this effect in neurons but was too brief to cause voltage-dependent Ca\textsuperscript{2+} release in the megakaryocyte. Application of this strong predepolarization had no significant effect on the voltage-dependent Ca\textsuperscript{2+} increase evoked by a 4-s, 80-mV depolarization from −75 mV; the average peak Ca\textsuperscript{2+} increase was 228 ± 19 nM for control steps versus 212 ± 30 nM for steps applied 4.5 ms after the predepolarization; \( n = 5, p < 0.05 \). Overall, therefore, these data argue against a role for dihydropyridine receptors or other voltage-gated Ca\textsuperscript{2+} channels or shifts of G-protein \beta\gamma subunit binding in the voltage sensor responsible for controlling Ca\textsuperscript{2+} signaling via GPCRs.

Requirement of P2Y\textsubscript{1} Receptors for the Voltage-dependent Ca\textsuperscript{2+} Release Induced by ADP and Thimerosal but Not Other G\textsubscript{q}-coupled Receptors—ADP is known to stimulate platelets via two G-protein-coupled receptors, P2Y\textsubscript{1} and P2Y\textsubscript{12}, which interact synergistically to generate full platelet aggregation responses (44–46). ADP-evoked Ca\textsuperscript{2+} mobilization in the platelet has been reported to be due either entirely to P2Y\textsubscript{1} receptors (17, 47, 48) or to also involve P2Y\textsubscript{12} receptors (49–51). We, therefore, turned to P2Y\textsubscript{1} receptor-deficient (P2Y\textsubscript{1}−/−) mice to assess the relative role of different P2Y receptors in the ADP-

Fig. 2. Membrane depolarization fails to stimulate Ca\textsuperscript{2+} mobilization during direct elevation of phospholipase C products or after receptor-dependent activation of phospholipase C\textsubscript{Y1}[Ca\textsuperscript{2+}], recordings (fluor-3/fluor ratio) during photolytic release of IP\textsubscript{3} (A), G-PIP\textsubscript{2} (B), or IP\textsubscript{3} in the presence of 100 \mu M OAG (C) are shown. The cell membrane potential was clamped at either −75 or 5 mV as indicated by the lower panels. A and C show two traces from different sections of the same experiment in which the potential was −75 mV throughout (black traces) or was stepped to 5 mV for 2 s after the uncaging event (red). Arrows indicate application of a brief (~2 ms) flash of UV illumination at a constant intensity for each experiment. D, stimulation of phospholipase C\textsubscript{Y1}-coupled PDGF receptors fails to induce voltage-dependent Ca\textsuperscript{2+} release.

2D). Again, this supports a location for the voltage sensor upstream of phosphatidylinositol 4,5-bisphosphate hydrolysis or IP\textsubscript{3} and DAG-dependent events.
dependent depolarization-evoked $Ca^{2+}$ response in the megakaryocyte. ADP induced a $Ca^{2+}$ increase ($344 \pm 47$ nM, $n = 26$) and a robust voltage-dependent $Ca^{2+}$ increase ($232 \pm 48$ nM, $n = 26$) in wild type mouse megakaryocytes (Fig. 4A). As shown previously for rat megakaryocytes (7), this effect of membrane potential is predominantly due to release of $Ca^{2+}$ from internal stores as it is observed in $Ca^{2+}$-free (Fig 4B; $n = 7$) and Na$^+$-free medium ($n = 12$, data not shown). P2Y$_1$ receptors were required for the $Ca^{2+}$ responses to both ADP (1 $\mu$M) and depolarization as they were absent in P2Y$_{1-/1-}$ mouse megakaryocytes (43 of 43 cells; see Fig. 4C). Depolarization-evoked $Ca^{2+}$ increases were still observed during exposure to U46619 and 5-HT in P2Y$_{1-/1-}$ megakaryocytes (Fig. 5, $A$ and $B$; $n = 15$ and 19, respectively); thus, functional G$_{q}$-coupled receptor responses remain intact in this genomic model. We have previously demonstrated that the thiol reagent thimerosal, which elevates $[Ca^{2+}]_i$ via both sensitization of IP$_3$ receptors and depletion of the internal stores (52), can induce a small voltage-dependent $Ca^{2+}$ release in rat megakaryocytes (12). This response was also observed in the mouse megakaryocyte (Fig. 5C, $n = 7$) and can be attributed to the action of thimerosal on sulfhydryl groups, as it was reversed by the reducing agent dithiothreitol (Fig. 5C) (52). However depolarization-evoked $Ca^{2+}$ release was not observed in P2Y$_{1-/1-}$ megakaryocytes during the response to thimerosal (Fig. 5D; $n = 19$), indicating that stimulation of P2Y$_1$ receptors, for example via constitutive or autocrine activation (53, 54), is necessary for the ability of thimerosal to induce the voltage-dependent response rather than via the sensitization of IP$_3$ receptors per se. Together with the lack of response to depolarization during stimulation of PDGF receptors or release of phospholipase C products (see earlier), this confirms that the principal voltage sensor is upstream of IP$_3$ production.

**Effect of Heterotrimeric G-protein Activation by Intracellular GTPγS or Aluminum Tetrafluoride—Intracellular application**

**FIG. 3.** Potentiation of P2Y$_1$ receptor $Ca^{2+}$ responses is maintained in the presence of nifedipine and is weakly dependent upon holding potential. $A$, representative trace showing the ability of depolarization ($80 \text{mV}, 10$-s duration) to potentiate ADP-evoked $Ca^{2+}$ mobilization in the presence of nifedipine ($50$ $\mu$M). The whole-cell current is also shown in $A$ (middle trace) to demonstrate the inhibition of voltage-gated $K^+$ current by nifedipine. $B$, average peak $[Ca^{2+}]_i$ increases (5–17 cells, as indicated) evoked by 80-mV, 10-s duration depolarizations in the absence of agonist, in the presence of 1 $\mu$M ADP, or in the presence of both 1 $\mu$M ADP and $50$ $\mu$M nifedipine. Nifedipine was applied for either 3–5 min (Nif (short)) or $>20$ min (Nif (long)) before depolarization. $C$, average peak $[Ca^{2+}]_i$ increases evoked by 80-mV, 10-s-duration depolarizations from different holding potentials ($V_{\text{hold}}$; $n = 5–19$ cells for each point). Cells were held at each holding potential throughout application of agonist and depolarization.

**FIG. 4.** P2Y$_1$ receptors are essential for induction of voltage-dependent $Ca^{2+}$ release by ADP. $[Ca^{2+}]_i$ responses of wild type (WT; $A$ and $B$) and P2Y$_{1-/1-}$ mouse megakaryocytes (C) to 1 $\mu$M ADP and 10-s step depolarizations from $-75$ to $+5$ mV in standard external saline ($A$ and $C$) or $Ca^{2+}$-free medium ($B$).
of the poorly hydrolysable GTP analogue, GTPγS, is frequently employed as a tool to activate heterotrimeric G-proteins in the absence of agonist (55). In wild type murine megakaryocytes, the addition of 50 μM GTPγS to the patch pipette induced irregular spikes of [Ca^{2+}]_i increase during whole-cell recordings (Fig. 6Ai; n = 8). Application of 10-s duration pulses from −75 to +5 mV significantly potentiated the GTPγS-induced Ca^{2+} increases (5.7-fold increase in the integral of the Ca^{2+} increase above the base line; n = 6, p < 0.01; Fig. 6, Aii and B). This effect of depolarization was not the result of sustained autocrine activation of P2Y₁ or thromboxane A₂ receptors as it was maintained during continuous perfusion with agonist-free saline and was also observed in separate experiments in the presence of either the nonspecific nucleotidase apyrase (0.32 units ml⁻¹, n = 7) or inhibition of thromboxane A₂ production by aspirin (100 μM; n = 6). Interestingly, the [Ca^{2+}]_i responses to 50 μM GTPγS (50 μM) both with (n = 9) or without (n = 5) depolarization were eliminated in P2Y₁⁻/⁻ megakaryocytes (Fig. 6, A, iii and iv). Although a small [Ca^{2+}]_i increase was observed at a higher concentrations of GTPγS (400 μM) in P2Y₁⁻/⁻ megakaryocytes, this was associated with an increased inward membrane current, making it difficult to further examine the effects of this activator of G-proteins. The above data do, however, show that depolarization-evoked Ca^{2+} increases induced by low levels of GTPγS require the presence of P2Y₁ receptors.

Although many electrophysiological studies have used GTPγS to activate heterotrimeric G-proteins and, thus, downstream effector pathways, [³²P]GTPγS binding is commonly used to assess the proportion of activated receptors (56). This is based on the fact that the GTP analogue binds essentially irreversibly to Ga subunits after receptor activation has promoted release of GDP. The effect of GTPγS on voltage-dependent Ca^{2+} mobilization and its absence in P2Y₁⁻/⁻ megakaryocytes can, therefore, be explained by a higher background activation of P2Y₁ compared with other Ga₉-coupled receptors in the megakaryocyte. This does, however, limit the use of GTPγS as a tool for assessing an innate voltage dependence to heterotrimeric G-proteins. In contrast to GTPγS, AlF₄⁻ can bind to Ga subunits and activate downstream targets while GDP is still attached by mimicking the action of the γ phosphate of GTP (57, 58). Perfusion of AlF₄⁻ resulted in an increase in [Ca^{2+}]_i and small depolarization-evoked transients (Fig. 7A), although these were of far smaller amplitude compared with those observed in the presence of 1 μM ADP (70 ± 26 nM, n = 7, during AlF₄⁻ versus 232 ± 48 nM, n = 26, during ADP; p < 0.01). This effect of AlF₄⁻ was absent in P2Y₁⁻/⁻ megakaryocytes despite a small increase in [Ca^{2+}]_i, whereas voltage-dependent Ca^{2+} mobilization was observed during subsequent addition of thromboxane A₂ (Fig. 7B; representative of 6 cells). Therefore, the primary site of action of membrane voltage in the control of GPCR-dependent Ca^{2+} mobilization is upstream of the heterotrimeric G-proteins.

**DISCUSSION**

This study provides further evidence that changes in the cell membrane potential can markedly regulate Ca^{2+} signaling via a number of GPCRs coupled to Go₉ proteins (3, 5, 7, 9, 32). The
Voltage Control of \(G_q\)-coupled Receptors

**Fig. 6.** \(Ca^{2+}\) mobilization induced by GTP\(\gamma\)S is voltage-dependent via a mechanism requiring P2Y\(_1\) receptors. A, intracellular application of 50 \(\mu\)M GTP\(\gamma\)S induced irregular transient \([Ca^{2+}]_i\) increases at a constant holding potential of \(-75\) mV (i). This \([Ca^{2+}]_i\) response was markedly potentiated by a train of 10-s duration steps to +5 mV at 0.05 Hz (ii); note that this voltage paradigm was occasionally interrupted to update capacitance and series resistance compensation. In P2Y\(_1^{+/−}\) megakaryocytes GTP\(\gamma\)S was unable to evoke \([Ca^{2+}]_i\) increases either alone (iii) or in combination with depolarizing pulses (iv). Traces in (i–iv) have the same timescale. B, the \([Ca^{2+}]_i\) increase, measured as the integral (nM s) of the response above base line, is shown for GTP\(\gamma\)S alone \((n = 8, \text{black bar})\) or in combination with steps to +5 mV \((n = 6, \text{white bar})\). \(*\ast, p < 0.01\). WT, wild type.

\([Ca^{2+}]_i\), response to a step depolarization during exposure to different agonists suggests a rank order for voltage dependence of ADP > thromboxane A\(_2\) > 5-HT, which activate \(Ca^{2+}\) release in the platelet/megakaryocyte lineage via P2Y\(_1\), TP\(_\alpha\), and 5-HT\(_2A\) receptors, respectively (17, 29, 30, 59, 60). All are class A GPCRs (2), which contribute the largest (\(\approx 90\%\)) of all known members of this superfamily. It is, therefore, likely that signaling via many other receptors within this family will show the potentially important phenomenon of voltage dependence. It was interesting that the depolarization-evoked \([Ca^{2+}]_i\) increases in the megakaryocyte were often transient, particularly after the larger responses induced during exposure to ADP (see for example Fig. 1A). In part, this reflects the tendency for IP\(_3\)-dependent \(Ca^{2+}\) mobilization to generate oscillatory rather than sustained \([Ca^{2+}]_i\), increases in the megakaryocyte (14). Indeed, depolarization induces oscillatory \(Ca^{2+}\) responses in a proportion of cells, particularly with longer duration pulses (8). However, the transient responses to voltage could also result from an underlying ability of the voltage sensor to detect changes in membrane potential more than absolute transmembrane voltage. This may be beneficial because it would promote the detection of action potential frequency in excitable tissues. Further work is required to address this issue.

Evidence from several studies in the megakaryocyte and coronary artery smooth muscle are consistent with the hypothesis that depolarization stimulates IP\(_3\) production during activation of P2Y\(_1\) and other \(G_q\)-coupled receptors (5, 7–9, 12, 61). Thus, the voltage-dependent \([Ca^{2+}]_i\) increase is predominantly due to IP\(_3\)-dependent \(Ca^{2+}\) release with associated \(Ca^{2+}\) influx via second messenger-dependent or store-dependent pathways. It is always possible that some other as yet undetermined \(Ca^{2+}\)-conducting pathway is also involved; however, we have previously ruled out a requirement for the one other obvious pathway, namely Na\(^+\)/\(Ca^{2+}\) exchange (7). The fundamental role of IP\(_3\)-dependent \(Ca^{2+}\) release explains the essential requirement for functional IP\(_3\) receptors for the response to volt-
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in the absence of exogenous agonist (see for example Fig. 1A, summarized in Fig. 1E), which we attribute to activation of P2Y<sub>1</sub> receptors, as this response was completely abolished in megakaryocytes from P2Y<sub>1</sub>&#8722;/&#8722; mice.<sup>3</sup> Unlike GTPγS, AIF<sub>4</sub><sup>-</sup> is able to activate G-proteins with GDP bound (57, 58) and, therefore, generates a [Ca<sup>2+</sup>]<sub>i</sub> increase with little delay (see Fig. 7). Basal activity within the P2Y<sub>1</sub>, but not other G<sub>q</sub>,-coupled receptors, can also account for the ability of thimerosal to induce a small voltage-dependence in wild type but not P2Y<sub>1</sub>&#8722;/&#8722; megakaryocytes. The thiol reagent is still able to elevate [Ca<sup>2+</sup>]<sub>i</sub> in P2Y<sub>1</sub>&#8722;/&#8722; megakaryocytes due to its additional action as an inhibitor of endomembrane Ca<sup>2+</sup>-ATPases (52). However, to completely explain the sustained voltage dependence during GTPγS activation in wild type cells and yet complete lack of the response to depolarization during AIF<sub>4</sub><sup>-</sup> or thimerosal in the P2Y<sub>1</sub>&#8722;/&#8722; megakaryocytes, depolarization must be able to stimulate P2Y<sub>1</sub> receptor activity in the absence of agonist. This is consistent with the emerging hypothesis that the receptor, G-protein, and effector molecules form a stable complex rather than dissociating during activation (65–68). Thus, an innate voltage dependence to the receptor can be transferred through the transduction cascade but only when the GPCR is present.

A sensitivity of the GPCR to the transmembrane potential rather than the G-protein or phospholipase C can be predicted based upon the fact that the receptor is the only protein within the transduction cascade that spans the plasma membrane.

The voltage dependence could result from configurational changes in charged residues within the region of the receptor that influence G-protein activation or that control agonist binding. Additionally, voltage dependence to binding of a polar or charged agonist could contribute in an analogous manner to unblock of N-methyl-D-aspartate receptors by removal of bound Mg<sup>2+</sup> (69). During application of exogenous agonist, voltage may act both directly on the receptor and via alterations of agonist binding; however, only the former mechanism can contribute to the potentiation of GTPγS-evoked Ca<sup>2+</sup> release by depolarization since this was maintained after measures that reduce autocrine activation such as constant perfusion, exonucleotidases, or aspirin treatment. Furthermore, other measures that are equally effective at inducing secretion, such as phorbol ester and elevation of IP<sub>3</sub>, failed to promote voltage-dependent Ca<sup>2+</sup> release (see Fig. 2). For the P2Y<sub>1</sub> receptor, the regions controlling interactions with its G-proteins are undefined; however, mutagenesis has indicated a number of charged residues within the transmembrane-spanning regions that markedly influence activation by 2-<wbr/>MeSADP (70). These residues are postulated to be involved in agonist recognition and, thus, could be involved in a voltage dependence to ligand binding. The modulation of Ca<sup>2+</sup> release that we observe in the absence of agonist would be expected to involve charged residues that control the efficiency with which the receptor activates G-proteins. Ben Chaim et al. (3) have recently suggested that m1 and m2 muscarinic receptors expressed in Xenopus oocytes are voltage-dependent, with depolarization exerting an opposite effect on these two receptors. From agonist binding experiments, the affinity of m2 receptors for acetylcholine is reduced, yet the affinity of m1 receptors for the agonist is enhanced by K<sup>+</sup>-induced depolarization. The authors argue that the voltage sensor resides outside the agonist binding site since this region is conserved for the two receptors and, therefore, propose that the sensor lies in the region that couples to the G-protein. We were unable to directly investigate whether membrane potential directly influences agonist binding at P2Y receptors since alterations of external K<sup>+</sup>, which would be required to set different membrane potentials in cell suspensions for radioligand binding studies, is able to modulate ADP-

![Figure 7](image_url)

**FIG. 7.** Stimulation of voltage-dependent Ca<sup>2+</sup> release by aluminun tetrafluoride also requires the presence of P2Y<sub>1</sub> receptors. Cells were exposed to the G-protein activator AIF<sub>4</sub><sup>-</sup> using a mixture of 5 mM NaF and 100 μM AlCl<sub>3</sub>. A, exposure to AlF<sub>4</sub><sup>-</sup> reversibly induced a [Ca<sup>2+</sup>]<sub>i</sub> increase and voltage-dependent Ca<sup>2+</sup> mobilization. Subsequent application of 1 μM ADP shows the larger voltage-dependent Ca<sup>2+</sup> responses during P2Y<sub>1</sub> receptors. B, AlF<sub>4</sub><sup>-</sup> failed to induce voltage-dependent Ca<sup>2+</sup> mobilization in P2Y<sub>1</sub>&#8722;/&#8722; megakaryocytes, even in cells in which depolarization potentiated Ca<sup>2+</sup> responses induced by thromboxane A<sub>2</sub> receptor stimulation with U46619. The figures are representative of seven (A) and five (B) cells.
evoked Ca²⁺ mobilization under conditions where the membrane potential does not change.⁴

In conclusion, we have investigated the mechanism underlying the ability of membrane depolarization to potentiate Ca²⁺ mobilization during activation of GPCRs coupled to Goq, which could have important consequences in a variety of cell types. The evidence supports the hypothesis that the principal voltage sensor lies at the level of the receptor rather than a downstream signaling event.

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⁴ Samantha J. Pitt, J. Martinez-Pinna, and M. P. Mahaut-Smith, unpublished observations.