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Differential Free Intracellular Calcium Release by Class II Antiarrhythmics in Cancer Cell Lines[§]

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ABSTRACT

Class II antiarrhythmics or β -blockers are antisympathetic nervous system agents that act by blocking β -adrenoceptors. Despite their common clinical use, little is known about the effects of β -blockers on free intracellular calcium (Ca_i^{2+}), an important cytosolic second messenger and a key regulator of cell function. We investigated the role of four chemical analogs, commonly prescribed β -blockers (atenolol, metoprolol, propranolol, and sotalol), on Ca_i^{2+} release and whole-cell currents in mammalian cancer cells (PC3 prostate cancer and MCF7 breast cancer cell lines). We discovered that only propranolol activated free Ca_i^{2+} release with distinct kinetics, whereas atenolol, metoprolol, and sotalol did not. The propranolol-induced Ca_i^{2+} release was significantly inhibited by the chelation of extracellular calcium with ethylene glycol tetraacetic acid (EGTA) and by dantrolene, an inhibitor of the endoplasmic

reticulum (ER) ryanodine receptor channels, and it was completely abolished by 2-aminoethoxydiphenyl borate, an inhibitor of the ER inositol-1,4,5-trisphosphate (IP_3) receptor channels. Exhaustion of ER stores with 4-chloro-*m*-cresol, a ryanodine receptor activator, or thapsigargin, a sarco/ER Ca_i^{2+} ATPase inhibitor, precluded the propranolol-induced Ca_i^{2+} release. Finally, preincubation of cells with sotalol or timolol, nonselective blockers of β -adrenoceptors, also reduced the Ca_i^{2+} release activated by propranolol. Our results show that different β -blockers have differential effects on whole-cell currents and free Ca_i^{2+} release and that propranolol activates store-operated Ca_i^{2+} release via a mechanism that involves calcium-induced calcium release and putative downstream transducers such as IP_3 . The differential action of class II antiarrhythmics on Ca_i^{2+} release may have implications on the pharmacology of these drugs.

Introduction

Class II antiarrhythmics or β -blockers have been in clinical use for the treatment of cardiovascular conditions such as angina and hypertension for more than five decades (Black et al., 1964; Chobanian et al., 2003). The cardioprotective effects of class II antiarrhythmics are linked to the inhibition of β -adrenoceptor (β -AR) signaling. There are three types of β -ARs: β_1 -AR, found mainly in cardiac cells; β_2 -AR, present in bronchial and vascular tissue; and β_3 -AR, largely expressed in adipose tissue. β -ARs are also expressed in many primary and metastasized tumors (Daly and McGrath, 2011; Cole and Sood, 2012).

Upon stimulation by catecholamines, β -AR signaling results in elevated cAMP levels and activation of the cAMP-dependent protein kinase A (Naga Prasad et al., 2001; Cole and Sood,

2012), which targets L-type Ca^{2+} channels ($\text{Ca}_v1.2$), activating a calcium influx (Weiss et al., 2013). Few reports have analyzed the direct effects of β -blockers on free intracellular calcium (Ca_i^{2+}) although previous studies have suggested that there is a reduction in the levels of free Ca_i^{2+} in platelets and erythrocytes of hypertensive patients treated with β -blockers (Erne et al., 1984; Baumgart et al., 1986).

Free Ca_i^{2+} is a potent second messenger that regulates many different cellular processes, including cell proliferation, cell differentiation, gene transcription, and apoptosis (Berridge et al., 2000; Carafoli et al., 2001). The level of cytosolic-free calcium (~ 100 nM) is tightly regulated, and most Ca_i^{2+} resides in intracellular stores, which in nonmuscle cells are principally located in the endoplasmic reticulum (ER) (Berridge et al., 2003). Ca_i^{2+} can be released from the ER through ryanodine receptor (RyR) (Zorzato et al., 1990) and inositol-1,4,5-trisphosphate (IP_3) receptor channels (Nixon et al., 1994). Calcium transients resulting from Ca^{2+} influx or Ca_i^{2+} release give rise to fast Ca^{2+} spikes or slower oscillatory waves that, depending on their kinetics and amplitude, translate to different cellular functions (Berridge et al., 2003).

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ABBREVIATIONS: 2-APB, 2-aminoethoxydiphenyl borate; β -AR, β -adrenoceptor; Ca_i^{2+} , free intracellular calcium; CICR, calcium-induced calcium release; 4-CmC, 4-chloro-*m*-cresol; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; IP_3 , inositol-1,4,5-trisphosphate; MPRC, membrane potential regulating compound; PBS, phosphate-buffered saline; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; TRP, transient receptor potential channels.

Despite the critical role of free Ca_i^{2+} as a regulator of cell function, the effects of β -blockers on the mobilization and kinetics of Ca_i^{2+} have received limited attention. Some reports regarding the regulation of calcium by β -blockers exist and include the investigations of Ca_i^{2+} levels in different disease models of heart failure or hypertension (Doi et al., 2002; Reiken et al., 2003; Tuncay et al., 2013; Cseplo et al., 2016). Other studies have examined the effects of β -blockers on β -adrenergic mediated calcium entry (e.g., after activation with β -AR agonists, such as isoproterenol or albuterol) and their role on cell contraction and vasorelaxation in different tissues and cell types (Sakanashi and Takeo, 1983; Yao et al., 2003; Priviero et al., 2006; Shahbaz et al., 2011; Cekic et al., 2013; Keller et al., 2014) (see Supplemental Table 1 for details). Surprisingly, the direct activation of free Ca_i^{2+} release by β -blockers has not been examined before in excitable or nonexcitable cells.

We have previously shown that several membrane potential regulating compounds (MPRCs), including the antiarrhythmics amiodarone and dofetilide, activate store-operated Ca_i^{2+} release in mammalian cancer cells (Petrou et al., 2017). Several epidemiologic studies have reported that the use of β -blockers correlates with a lower incidence of cancer progression and mortality for prostate (Perron et al., 2004; Grytli et al., 2013), breast (Powe et al., 2010; Barron et al., 2011; Melhem-Bertrandt et al., 2011), and skin cancers (De Giorgi et al., 2011; Lemeshow et al., 2011; De Giorgi et al., 2017).

We asked whether β -blockers could activate Ca_i^{2+} release in cancer cells. We concentrated on four commonly used β -blockers, including two β_1 -selective β -blockers—atenolol and metoprolol—and two nonselective β -blockers—propranolol and sotalol. We used PC3 and MCF7, prostate and breast cancer cell lines, respectively, to measure Ca_i^{2+} release by ratiometric live calcium imaging. In addition, we used a medium throughput patch-clamp system to measure ionic currents in the cells. The results show that 1) *only* propranolol activated a Ca_i^{2+} release, with distinct kinetics and amplitude; 2) the propranolol activation of Ca_i^{2+} stores was mediated by calcium-induced calcium release (CICR); and 3) the four β -blockers regulate endogenous whole-cell currents in cancer cell lines. Our results show differential activation of calcium stores and free Ca_i^{2+} release by several class II antiarrhythmics in nonexcitable, cancer cells and may have important implications for the mechanism of action and pharmacology of these β -blockers.

Materials and Methods

Compounds. All β -blockers were purchased from Sigma-Aldrich (Gillingham, UK). Stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) for atenolol and propranolol or in phosphate buffer saline (PBS), pH 7.4, without Ca^{2+} or Mg^{2+} (cat. no. 10010; Gibco ThermoFisher, Loughborough, UK) for metoprolol and sotalol according to the manufacturer's instructions. The following are the systematic names for the β -blockers: atenolol (cat. no. A7655), (\pm)-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]benzeneacetamide; metoprolol tartrate (cat. no. M5391), (\pm)-1-(isopropylamino)-3-[p-(β -methoxyethyl)-phenoxy]-2-propanol (+)-tartrate salt; propranolol hydrochloride (cat. no. P8688): (*S*)-1-isopropylamino-3-(1-naphthyl)-2-propanol hydrochloride; and sotalol hydrochloride (cat. no. S0278), N-[4-[1-hydroxy-2-(isopropylamino)ethyl]phenyl]methanesulfonamide hydrochloride (see Supplemental Fig. 1 for chemical structures). For all β -blockers, two different lots were purchased and tested in our experiments. Loxapine (cat. no. L106; Sigma-Aldrich), previously shown to activate store-operated

free Ca_i^{2+} release via CICR in cancer cells (Petrou et al., 2017), was used as a positive control for some live Ca_i^{2+} imaging experiments. Timolol (cat. no. T6394; Sigma-Aldrich) was used as an additional nonselective β -blocker to investigate the contribution of β -ARs to Ca_i^{2+} release. Ethylene glycol tetraacetic acid (EGTA), dantrolene, 4-chloro-*m*-cresol (4-CmC), thapsigargin (Sigma-Aldrich), and 2-aminoethoxydiphenyl borate (2-APB; Tocris Bioscience, Abingdon, UK) were also used in some experiments (see later). Stock solutions were prepared in DMSO (dantrolene, 2-APB, and thapsigargin), ethanol (4-CmC), or PBS (EGTA).

Cell Culturing. PC3 prostate cancer and MCF7 breast cancer cell lines were obtained from the American Type Culture Collection (Teddington, UK). Details of cell culture procedures have been described elsewhere (Thrasivoulou et al., 2013; Petrou et al., 2017). Briefly, cells were maintained in RPMI 1640 medium (Gibco ThermoFisher) supplemented with 10% fetal bovine serum and 5 mM L-glutamine and cultured at 37°C in a humidified incubator with 5% CO_2 and 21% O_2 atmosphere. For live calcium imaging experiments, 10^5 cells were seeded in 35-mm FluoroDishes (World Precision Instruments, Hitchin, UK), and experiments were performed in at least four to eight different passages (passage numbers 23–38 for PC3 cells and 35–44 for MCF7 cells).

Intracellular Live Calcium Imaging. Free Ca_i^{2+} release was measured as a change in the ratio of Fluo-4/FuraRed (free calcium/bound calcium) over time. The two indicators have reciprocal shifts in intensity owing to calcium binding and are used together in a ratiometric probe strategy described previously (Wang et al., 2010b; Thrasivoulou et al., 2013; Petrou et al., 2017). Briefly, cells were grown as a monolayer in 35 mm FluoroDishes for 3 to 4 days. Before imaging, the cells were incubated for 30–40 minutes at 37°C with the acetoxymethyl ester derivatives of the calcium indicators Fluo-4 and FuraRed (ThermoFisher Scientific) at 1.1 and 1.4 $\mu\text{g}/\text{ml}$, respectively. Cells were washed (3 \times) with and replaced in 1 ml PBS without Ca^{2+} or Mg^{2+} (Gibco ThermoFisher, as described) for live calcium imaging, performed using an Olympus FluoView FV100 confocal microscope (Olympus, UK) equipped with a 20 \times /0.75 NA objective and a temperature-controlled chamber at 37°C. Calcium indicators were excited with an argon laser at 488 nm, and fluorescence was recorded every 2.2 seconds in the green channel for Fluo-4 (500–580 nm) and in the red channel for FuraRed (630–730 nm). Confocal imaging was started and, after a baseline was achieved, Ca_i^{2+} release was measured by applying β -blockers to the FluoroDish as a bolus, at a volume of 0.5–5 μl , to achieve a final β -blocker concentration of 25, 50, 100, 150, or 250 μM ; vehicle controls were performed using a similar protocol. Data acquisition was performed using Olympus FV10-ASW 4.2 software.

Although the PBS used here is nominally Ca^{2+} free, the residual Ca^{2+} concentration in PBS was measured to be >60 μM in this solution (Petrou et al., 2017). In some experiments, 5 mM EGTA was added to the PBS used as imaging media to chelate free residual Ca^{2+} ; cells were preincubated for 5 minutes before imaging was started. We have previously shown that using 5 mM EGTA in PBS reduces the residual free Ca^{2+} to <10 nM (Petrou et al., 2017). In other experiments, for the pharmacologic characterization of the mechanisms of Ca_i^{2+} store activation, cells were preincubated with the following: 1) 10 μM dantrolene for 5 minutes at 37°C to inhibit the ER RyR channels (Zhao et al., 2001); 2) 1 mM 4-CmC for 12 minutes at 37°C to activate RyR channels and exhaust the Ca_i^{2+} ER stores (Zorzato et al., 1993); 3) varying concentrations (1, 25, 50, or 100 μM) of 2-APB for 10 minutes at 37°C to inhibit the ER IP_3 receptor channels (Maruyama et al., 1997); 4) 5 μM thapsigargin for 15–20 minutes at 37°C to discharge Ca_i^{2+} from the ER (Thastrup et al., 1990); or 5) 250 μM sotalol or timolol (nonselective β -blockers) for 2 minutes at 37°C for blockade of β -ARs (Baker, 2005).

Data Analysis and Statistics. Data from live calcium imaging experiments were analyzed for time kinetics and amplitude as described previously (Thrasivoulou et al., 2013) using a Mathematica script (Wolfram, Hanborough, UK) (Petrou et al., 2017). The kinetics of the calcium waveform were characterized by different time constants: rise time (time from baseline to peak), dwell time (duration of the plateau

phase), and fall time (time to return to baseline). The amplitude of the response was calculated as a fold increase in fluorescence intensity from baseline to peak ($\Delta F/F_0$, where $\Delta F = F - F_0$ and F is the maximum fluorescence intensity over basal level, F_0). Data were analyzed using the D'Agostino-Pearson test for normal distribution and the Mann-Whitney U test for statistical significance using MedCalc (Ostend, Belgium), and plotted using OriginPro 2016 (OriginLab, Northampton, MA).

Automated Medium-Throughput Electrophysiology. Endogenous whole-cell currents of PC3 cells in response to the application of β -blockers were measured using the QPatch automated cell patch-clamp system (Sophion Bioscience, Ballerup, Denmark), as described previously (Petrou et al., 2017). PC3 cells were cultured and harvested using Detachin (Genlantis, San Diego, CA) and kept in the QStirrer of the QPatch for up to 4 hours before the automatic preparation. The cells were transferred to the QFuge, centrifuged, and washed $2\times$ in extracellular solution (see following) before being applied to the measuring site in the QPlate of the QPatch. A pressure of -70 mBar was applied to obtain positioning and sealing of the cells, and a whole-cell protocol with pressure pulses at -150 mBar was used to obtain whole-cell formation. Gigaseals were formed upon execution of a combined suction/voltage protocol. The intracellular solutions and compounds were applied by eight pipettes. The intracellular solution contained (in millimolar concentrations): 5.3 CaCl_2 , 1.7 MgCl_2 , 10 EGTA, 10 HEPES, 120 KCl, and 4 $\text{Na}_2\text{-ATP}$; with pH 7.2 , osmolarity of 295 mOsm, and a calculated free calcium concentration of 680 nM. The extracellular solution contained (in millimolars): 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 4 KCl, and 145 NaCl, with pH 7.4 , and osmolarity adjusted to 285 – 295 mOsm. Currents were recorded using a command ramp from -120 to $+120$ mV at 0.5 mV/ms every 3 seconds, with a holding voltage of -10 mV between executions of the ramps. Data were sampled at 5 kHz and filtered using a fourth-order Bessel filter.

Six different concentrations of individual drugs were used: increasing concentrations of the β -blocker from 0 to 500 μM were applied sequentially on the same cell. The QPatch system implements fine microfluidic control of the drug delivery time, and complete solution changes were made within 500 milliseconds. Average currents at $+100$ and -100 mV were analyzed to explore the concentration dependence of the modulation of endogenous whole-cell currents by β -blockers; statistical significance was calculated with the Wilcoxon test (MedCalc). Current-voltage (I - V) curves of control versus β -blocker were also constructed. Additional analysis of medium-throughput data from QPatch was carried out using Matlab (Mathworks, Natick, MA).

Real-Time Polymerase Chain Reaction. Total RNA was purified from PC3 cells by using the RNeasy Plus kit (Qiagen, Manchester, UK) and reverse-transcribed with the Omniscript RT kit (Qiagen), according to the manufacturer's instructions. Polymerase chain reaction was performed with the SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA) using a Bio-Rad (Watford, UK) CFX384 thermocycler. Expression levels of the genes of interest were normalized to GAPDH or β -actin, and melting curves were analyzed using Bio-Rad CFX Manager to verify the products. The following primers were purchased from Sigma-Aldrich: β_1 -AR (ADRB1) sense $5'$ -TACGGCTCCTTCTCTGCGA- $3'$ and antisense $5'$ -CAGGTACACGAAGGCCATGAT- $3'$; β_2 -AR (ADRB2) sense $5'$ -CATTGAGACCCTGTGCGTGA- $3'$ and antisense $5'$ -AGGGCTTGTGCTCCTTCAA- $3'$; β_3 -AR (ADRB3) sense $5'$ -GTTTTCTGGTGGCTACGC- $3'$ and antisense $5'$ -CCTAGCCAGTTCAGGGCAAG- $3'$; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense $5'$ -CGGATTTGGTTCGTATTGGGC- $3'$ and antisense $5'$ -TGGTCATGAGTCCTTCCACG- $3'$; β -actin (ACTB) sense $5'$ -CTGTGCTATCCCTGTACGCC- $3'$ and antisense $5'$ -ATCTTCATTGTGCTGGGTGCC- $3'$ (annealing at 60°C for 40 cycles; 70 melting curve reads off, from 60 to 95°C).

Results

Propranolol Activates Ca_i^{2+} Release in Cancer Cells with Distinct Kinetics but Other β -Blockers Do Not. The addition of 50 μM propranolol to the extracellular solution

caused a change in the ratio of Fluo-4/FuraRed in PC3 cells (Fig. 1), indicating release of free Ca_i^{2+} ; there was no observable Ca_i^{2+} release in response to atenolol, metoprolol, and sotalol (Fig. 2A). Similar results were observed in MCF7 breast cancer cells, indicating that this phenomenon is not cell line specific (Fig. 2B). We also tested various concentrations of β -blockers (Fig. 3) within the pharmacologic range (Joint Formulary Committee, 2017).

Three of the β -blockers used did not induce free Ca_i^{2+} release in either PC3 or MCF7 cancer cell lines (Fig. 2). We performed additional validation experiments to exclude the possibility that the lack of Ca_i^{2+} release by atenolol, metoprolol, and sotalol may have been due to cellular or technical factors. First, to confirm that the cells remained responsive to free Ca_i^{2+} release, loxapine, a compound previously shown to induce Ca_i^{2+} release in cancer cells (Petrou et al., 2017), was used as a positive control. In these experiments, atenolol, metoprolol, or sotalol were first added to the cells for 10 minutes, followed by loxapine in live calcium imaging experiments. There was no Ca_i^{2+} release in response to the three β -blockers ($n = 3$); however, the addition of loxapine to the *same* cells activated Ca_i^{2+} release (Fig. 4). Second, we procured different lots for the three drugs from the vendor (Sigma-Aldrich) and tested these in live Ca_i^{2+} imaging experiments. No Ca_i^{2+} release occurred in response to addition of any of the different lots of atenolol, metoprolol, or sotalol. The effect of the solvent vehicle used for drug suspension (either DMSO or ethanol) was tested and was found to have no effect on any of the responses (i.e., only propranolol, but not atenolol, metoprolol, or sotalol, induced μCa_i^{2+} release) (Supplemental Fig. 2).

The waveform of Ca_i^{2+} release was used to measure the amplitude and the time kinetics (rise, dwell, and fall times) as described elsewhere (Thrasivoulou et al., 2013). Figure 5 shows the distinct time constants of the Ca_i^{2+} release in response to 50 μM propranolol in PC3 and MCF7 cells; similar kinetics were observed in both cell lines.

Role of Calcium-Induced Calcium Release Mechanism in the Propranolol-Induced Ca_i^{2+} Release. We tested the hypothesis that CICR is the mechanism by which propranolol-induced Ca_i^{2+} release occurs in cancer cells. Four sets of experiments were performed. First, we added 5 mM EGTA to chelate the residual Ca^{2+} (>60 μM in the PBS used for calcium imaging experiments), as it is known that even micromolar levels of extracellular Ca^{2+} can activate CICR pathways (Berridge et al., 2003; Endo, 2009). The addition of 5 mM EGTA, which chelates extracellular Ca^{2+} concentration to <10 nM (Petrou et al., 2017), significantly inhibited the propranolol-induced Ca_i^{2+} release (Fig. 6).

Second, we used dantrolene and 4-CmC, modulators of RyR channels found in the ER (Zorzato et al., 1993; Zhao et al., 2001), to determine whether the ER calcium stores were activated in response to extracellular addition of propranolol. Incubation with 10 μM dantrolene, an inhibitor of RyRs, significantly inhibited the Ca_i^{2+} release induced by propranolol (Fig. 6). Incubation of cells with 1 mM 4-CmC, an activator of RyRs that is known to induce Ca_i^{2+} stores (Supplemental Fig. 3A), also inhibited the propranolol-induced Ca_i^{2+} release (Fig. 6).

Third, we used 2-APB, an inhibitor of IP_3 -induced Ca_i^{2+} release from the IP_3 receptor channels located in the ER (Maruyama et al., 1997) and also known to inhibit various

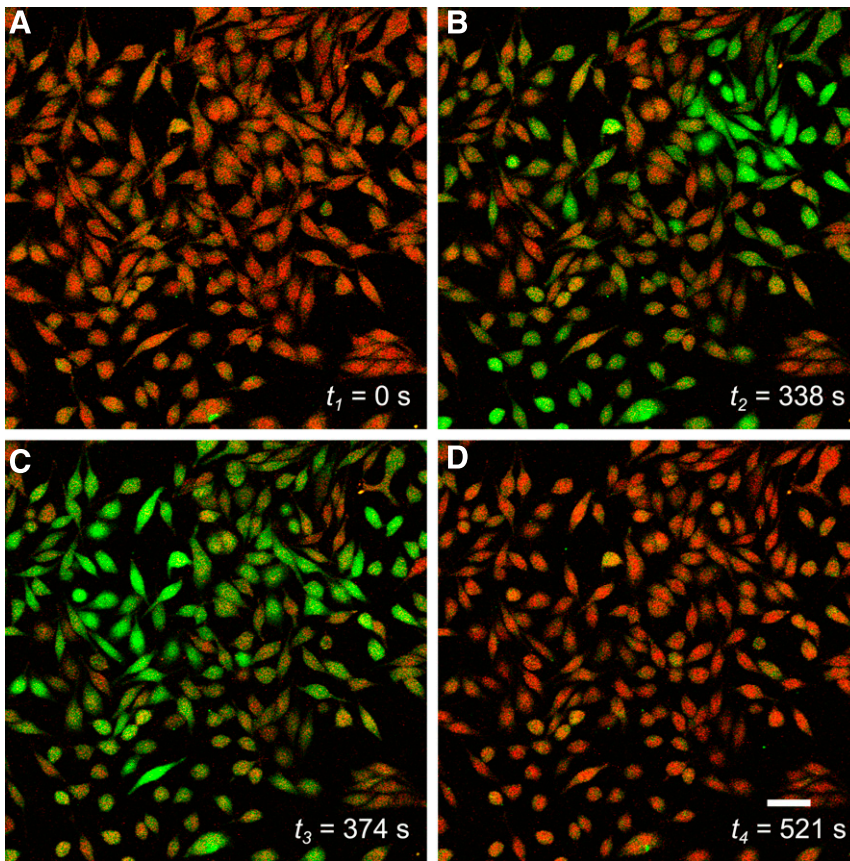


Fig. 1. Intracellular calcium (Ca_i^{2+}) release in PC3 cells in response to $50 \mu\text{M}$ propranolol. PC3 cells were loaded with the calcium indicators Fluo-4 (green fluorescence, an indicator of free calcium) and FuraRed (red fluorescence, an indicator of bound calcium) and monitored by time-lapse video microscopy every 2.2 seconds using an Olympus FluoView FV100 confocal microscope. Representative snapshots of free Ca_i^{2+} release at four time points (A–D) are shown: (A) Baseline before the addition of propranolol; (B) approximately 30% of cells in the observable frame undergoing Ca_i^{2+} release at $t = 338$ seconds, as observed by the simultaneous increase in Fluo-4 fluorescence and decrease in FuraRed signal; (C) peak of the Ca_i^{2+} release response ($t = 374$ seconds) with almost all cells showing a response (green); and (D) cells returning to baseline after the propranolol-induced Ca_i^{2+} release. Scale bar, $50 \mu\text{m}$, original magnification, $20\times$.

TRP ion channels (Xu et al., 2005; Togashi et al., 2008). Incubation with $50 \mu\text{M}$ 2-APB, a concentration within the range of IC_{50} values for the inhibition of IP_3 -induced Ca_i^{2+} release (Maruyama et al., 1997; Bootman et al., 2002; Saleem et al., 2014), also abolished the propranolol-induced Ca_i^{2+} release (Fig. 6). Similar results were observed when cells were incubated with 25 and $100 \mu\text{M}$ 2-APB (Supplemental Fig. 4).

Fourth, incubation with $5 \mu\text{M}$ thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase or SERCA (Thastrup et al., 1990), completely abolished the propranolol-induced Ca_i^{2+} release (Fig. 6). Thapsigargin, like 4-CmC, discharges the Ca_i^{2+} from thapsigargin-sensitive stores (e.g., the ER; Supplemental Fig. 3B) and has been previously used to investigate ligand-induced Ca_i^{2+} release (Thrasivoulou et al., 2013). In summary, these results indicate that the propranolol-induced Ca_i^{2+} release is likely to be a CICR-facilitated mechanism in which extracellular calcium contributes to the activation of Ca_i^{2+} release from the ER through RyR channels and IP_3 receptor channels.

β -ARs and Propranolol-Induced Ca_i^{2+} Release. The involvement of β -ARs in general for the activation of Ca_i^{2+} release by propranolol was investigated by using two different nonselective β -blockers: sotalol and timolol. We tested the hypothesis that blocking β -ARs using the known nonselective β -blockers would interfere with the propranolol-induced Ca_i^{2+} release. Neither sotalol nor timolol activated Ca_i^{2+} release in PC3 cells, and this was further confirmed by treating the same cells with loxapine (Fig. 4C; Supplemental Fig. 5); however, incubating the cells with either sotalol or timolol significantly inhibited the propranolol-induced Ca_i^{2+} release

(Fig. 7A), suggesting that propranolol exerts its function via the β -ARs. The Ca_i^{2+} release induced by loxapine (a dibenzoxazepine) remained unaffected by preincubation with sotalol or timolol (Fig. 7B).

Electrophysiological Characteristics of β -Blockers on Medium-Throughput Whole-Cell Currents in PC3 Cells. The electrophysiological characteristics of the four β -blockers on the endogenous currents in nonexcitable cancer cells, are not known (see Supplemental Table 2 for details on the electropharmacology of β -blockers in other cell types). We sought to establish a basic characterization of the effects of β -blockers on the endogenous whole-cell currents in PC3 cells using medium-throughput recording. Six concentrations of each β -blocker were tested in the cells, based on the pharmacologic doses (Supplemental Fig. 1; Joint Formulary Committee, 2017) and the IC_{50} values described previously (Supplemental Table 2), following a voltage-clamp protocol with a command ramp from -120 to $+120$ mV. Figure 8 shows the concentration dependence of whole-cell current regulation by β -blockers in PC3 cells at positive ($+100$ mV) and a negative (-100 mV) potential. β -Blockers regulate whole-cell currents with distinct features (Fig. 8, A–D); atenolol inhibited whole-cell currents at positive, but not at negative potentials, and metoprolol activated currents at negative and positive potentials. Both these effects were concentration-dependent.

In contrast, propranolol enhanced whole-cell currents at both positive and negative potentials at concentrations $<8.4 \mu\text{M}$. At higher concentrations (i.e., 56 – $500 \mu\text{M}$), currents were inhibited, which we ascribe to nonspecific effects at these very high ($>56 \mu\text{M}$) levels of the drug. Sotalol did not cause significant alterations to endogenous currents at any of the

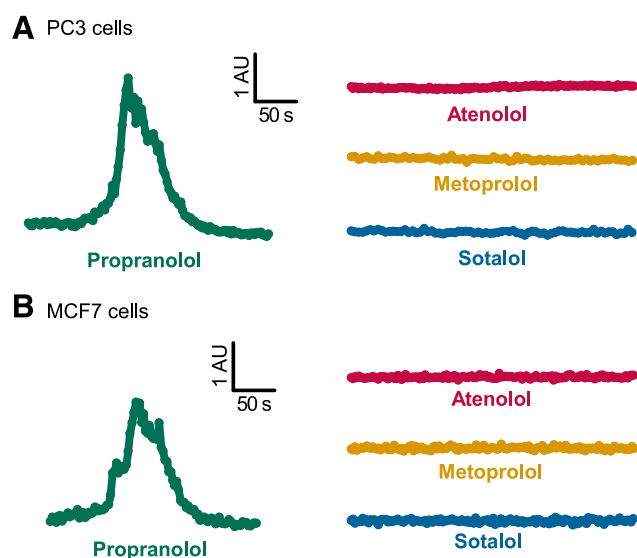


Fig. 2. Representative traces of Ca_i^{2+} release in (A) PC3 prostate cancer and (B) MCF7 breast cancer cells in response to the addition of $50 \mu\text{M}$ β -blockers: propranolol (green), atenolol (magenta), metoprolol (yellow), and sotalol (blue). The x -axis represents time (s) and the y -axis represents the Fluo-4/FuraRed ratio (free calcium/bound calcium, respectively) in arbitrary units (AU). The waveform was used to calculate the kinetics (rise, dwell, and fall times) of the Ca_i^{2+} release (see Fig. 5). The mean amplitude and time constants were used to select a representative trace for each compound ($n = 5$ – 8 experiments, $n = 151$ – 448 single-cell measurements per β -blocker). Of the four β -blockers tested, propranolol was the only one to induce Ca_i^{2+} release in both PC3 and MCF7 cell lines.

concentrations that we tested. The representative current-voltage (I-V) curves are shown in Fig. 8E. These results indicate that endogenous currents in PC3 cells can be modulated differently by the β -blockers investigated.

Discussion

In this study, we investigated the direct activation of calcium stores by atenolol, metoprolol, propranolol, and sotalol. We have shown that β -blockers have differential characteristics of Ca_i^{2+} mobilization in human cancer cell lines. Propranolol activates free Ca_i^{2+} release, whereas atenolol, metoprolol, and sotalol do not. We propose that CICR is a mechanism by which propranolol activates free Ca_i^{2+} release from intracellular stores.

Free Ca_i^{2+} is an important second messenger owing to its regulatory role of normal (Berridge et al., 2000) and malignant cell function (Prevarskaya et al., 2011). To the best of our knowledge, the regulation of Ca_i^{2+} mobilization by β -blockers in cancer cells remains largely unknown (Supplemental Table 1). We found that propranolol activates the release of Ca_i^{2+} from the cellular stores with distinct kinetics of rise, dwell, and fall times in both PC3 and MCF7 cancer cell lines. In comparison with previous research analyzing the kinetics of Ca_i^{2+} mobilization in response to Wnt ligands (Thrasivoulou et al., 2013) and other MPRCs in clinical use (Petrou et al., 2017), the propranolol-induced Ca_i^{2+} time constants (Fig. 5) suggest that this β -blocker activates a slow exhaustion and replenishment of Ca_i^{2+} stores, with a short dwell time (i.e., 20 ± 6 seconds, mean \pm S.D. of $n = 8$ imaging experiments).

The propranolol-induced Ca_i^{2+} release does not follow a classic pattern. A measurable Ca_i^{2+} release (i.e., a waveform with a well time of >15 seconds), is observed in around 30% of cells ($n = 518$ cells, from $n = 6$ experiments) at $35 \mu\text{M}$ propranolol, a variability reflected in the box plot (Fig. 3); however, at $50 \mu\text{M}$, $>98\%$ of cells show an increase in propranolol-induced Ca_i^{2+} release. The data suggest that activation of Ca_i^{2+} release in PC3 cells occurs between 35 and $50 \mu\text{M}$ (Fig. 3) with no significant, observable response to concentrations $<35 \mu\text{M}$. It should be noted that the Ca_i^{2+} release readout is not a direct assay of propranolol binding to effective receptors, which generally follows a classic Michaelis-Menten kinetics. The readout may reflect cooperativity within the numerous intermediate steps until a threshold is achieved to activate the intracellular calcium stores.

Extracellular calcium triggering CICR, an autocatalytic mechanism found in muscle (Endo, 2009) and nonmuscle cells (Verkhatsky and Shmigol, 1996; Petrou et al., 2017), is likely to be at least one of the signals that triggers the release of Ca_i^{2+} in response to propranolol. Our dantrolene results (Fig. 6A) suggest that type 1 and/or 3 RyRs are also likely to be involved in the calcium efflux from the ER (Zhao et al., 2001), although there are other RyRs inhibitors, such as ruthenium red (Xu et al., 1999), that we have not tested in our experiments. EGTA and dantrolene did not inhibit the propranolol-induced Ca_i^{2+} release completely (Fig. 6B); this may indicate that there are other mechanisms by which stores are activated by propranolol or may be due to a partial calcium chelation and RyR inhibition by these agents.

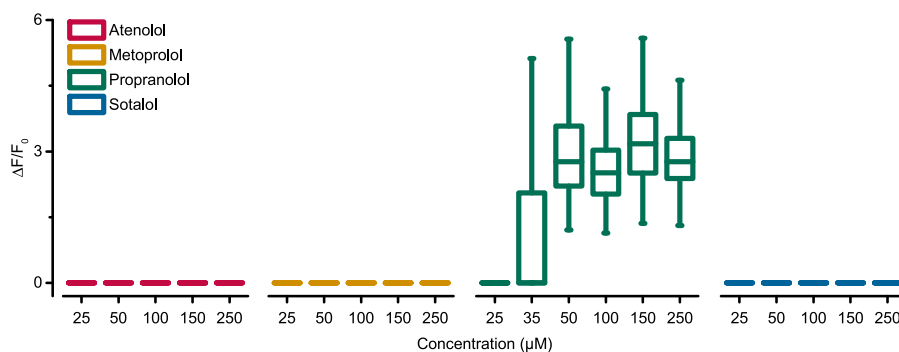


Fig. 3. Characterization of free Ca_i^{2+} release in response to various concentrations of β -blockers in PC3 cells. PC3 prostate cancer cells loaded with the calcium indicators Fluo-4 and FuraRed were treated with β -blockers, added as a bolus into the FluoroDish, and monitored over time using time-lapse confocal microscopy. Ca_i^{2+} release was measured as changes in the Fluo-4/FuraRed ratio; the amplitude of the Ca_i^{2+} release was calculated as fold increase in fluorescence intensity from baseline to peak ($\Delta\text{F}/\text{F}_0$) of the Fluo-4/FuraRed waveform and is presented as box plots. At a range of pharmacologic concentrations (see Supplemental Fig. 1 for details), only propranolol activated the release of Ca_i^{2+} . Other β -blockers, atenolol, metoprolol, and sotalol, did not mobilize Ca_i^{2+} ($n = 107$ imaging experiments and $n = 7256$ individual cells analyzed; with at least $n \geq 2$ experiments and $n > 130$ cells per concentration for each β -blocker).

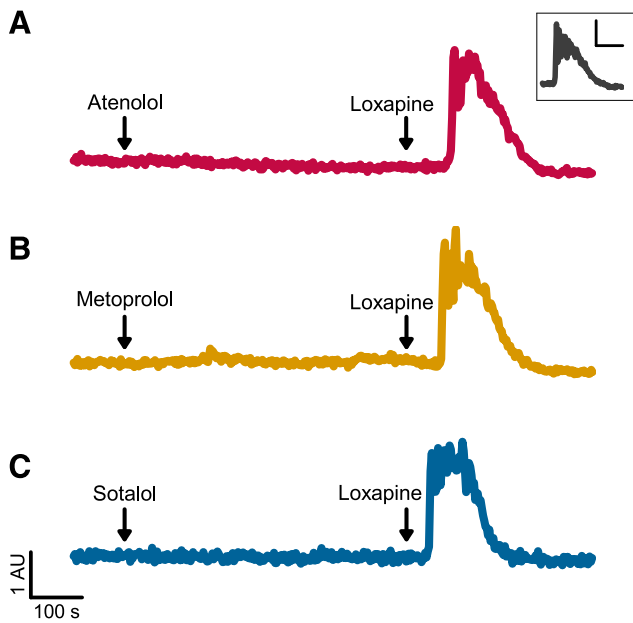


Fig. 4. Atenolol, metoprolol, and sotalol did not induce the release of Ca_i^{2+} in PC3 prostate cancer or MCF7 breast cancer cells. To confirm this observation, PC3 cells loaded with the calcium indicators Fluo-4 and FuraRed were treated with $50 \mu\text{M}$ β -blocker (atenolol, metoprolol, or sotalol, added as a bolus) and after 10 minutes of imaging, the cells were treated with $50 \mu\text{M}$ loxapine, a dibenzoxazepine antipsychotic drug known to activate the release of free Ca_i^{2+} in human cancer cells (see Fig. 4 from Petrou et al., 2017 for details). Representative traces of Ca_i^{2+} release (amplitude of the Fluo-4/FuraRed ratio over time) in response to the addition of (A) atenolol (magenta), (B) metoprolol (yellow), or (C) sotalol (blue), followed by the addition of loxapine; arrows indicate the time of addition ($n = 3$ per β -blocker, with $n = 161$ – 272 individual cells). A representative trace of loxapine-induced Ca_i^{2+} release in control cells (no previous treatments) is shown in the insert (scale: y-axis 1 AU and x-axis 100 seconds). Note that there was no Ca_i^{2+} release in response to any of the three β -blockers whereas loxapine caused an immediate release of Ca_i^{2+} in the cells.

Depletion of ER Ca_i^{2+} with thapsigargin, which inhibits the SERCA pump (Thastrup et al., 1990), or 4-CmC, which discharges the ER via activation of RyR channels (Zorzato et al., 1993; Herrmann-Frank et al., 1996), completely abolished the Ca_i^{2+} release induced by propranolol, suggesting that the ER is the main store from which calcium is released in response to this β -blocker (Fig. 6; Supplemental Fig. 3). We do not, however, exclude the possibility that other intracellular calcium stores (e.g., mitochondria) may also contribute to the propranolol-induced Ca_i^{2+} release (Michelangeli et al., 2005).

These data also suggest that IP_3 is involved in the propranolol-induced Ca_i^{2+} release. Here, 2-APB, an agent largely used as an

inhibitor of IP_3 -induced Ca_i^{2+} release (Maruyama et al., 1997; Choi et al., 2010; Saleem et al., 2014), abolished the Ca_i^{2+} release activated by propranolol (Fig. 6; Supplemental Fig. 4). 2-APB is also thought to block store-operated calcium entry channels (Gregory et al., 2001), the SERCA pump (Missiaen et al., 2001), and some members of the TRP family (Xu et al., 2005; Togashi et al., 2008), although these interactions are complex (Prakriya and Lewis, 2001; Xu et al., 2005) and vary across cell types (Bootman et al., 2002). It is possible that the blockade of TRP channels may also contribute to the inhibition of the propranolol-induced Ca_i^{2+} release caused by 2-APB, presuming that TRP channels may be involved in the activation of CICR pathways in response to propranolol (see later). Based on our observations, we suggest that inhibition of the propranolol-induced Ca_i^{2+} release by 2-APB indicates the involvement of IP_3 as an intracellular transducer that is produced upon propranolol-receptor binding and contributes to the activation of a calcium influx through IP_3 receptors in the ER, in agreement with our observations using thapsigargin since IP_3 -responsive Ca_i^{2+} pools are thapsigargin-sensitive (Tanaka and Tashjian, 1993; Tribe et al., 1994).

Class II antiarrhythmics are β -AR antagonists primarily, but they are also known to act upon potassium (Sakuta et al., 1992; Xie et al., 1998; Dupuis et al., 2005; Kawakami et al., 2006; Tamura et al., 2009) and sodium ion channels (Desaphy et al., 2003; Bankston and Kass, 2010; Wang et al., 2010a) (Supplemental Table 2). I–V curves of whole-cell patch-clamp recordings (Fig. 8E) showed the characteristics of an outward rectifying K^+ current, a current that has been previously described for PC3 cells (Laniado et al., 2001). Propranolol inhibits these endogenous currents similarly to those described for antiarrhythmic MPRCs, such as dofetilide (Petrou et al., 2017). At low concentrations (between 0.04 and $8.4 \mu\text{M}$), there is an increase in the inward and outward currents, which are inhibited at higher concentrations ($>56 \mu\text{M}$). In view of the absence of a measurable Ca_{2+i} release at $<35 \mu\text{M}$ propranolol, we speculate that this observation may reflect differences in the experimental design in which drugs are applied to the bath as a bolus for Ca_i^{2+} imaging compared with rapid microfluidic application in the QPatch recordings. The amplitude of the propranolol-induced Ca_i^{2+} release plateaus between 50 and $250 \mu\text{M}$ propranolol (Fig. 3), indicating that once the threshold concentration of $50 \mu\text{M}$ propranolol is reached, the Ca_i^{2+} stores are activated. The $50 \mu\text{M}$ propranolol is an order of magnitude greater than the concentration at which cell membrane currents are activated by propranolol (Fig. 8C). We have previously suggested that there may be a small number of

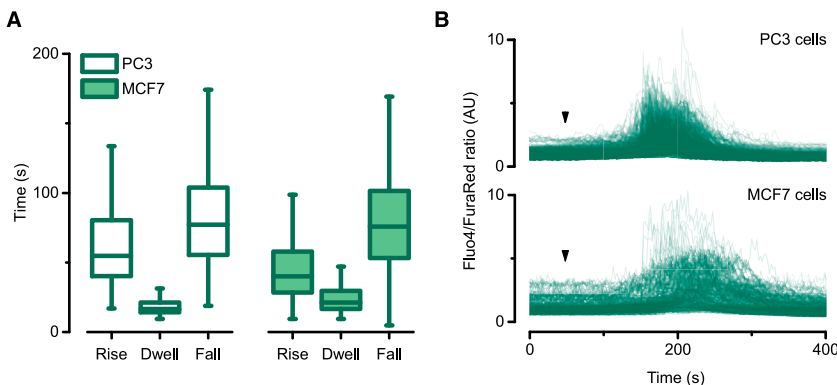


Fig. 5. Characterization of the propranolol-induced Ca_i^{2+} release kinetics in PC3 prostate cancer and MCF7 breast cancer cells. (A) The Ca_i^{2+} waveform generated by the addition of $50 \mu\text{M}$ propranolol to PC3 or MCF7 cells was defined by three time constants: rise, dwell, and fall times, represented as box plots ($n = 5$ – 8 imaging experiments per cell line, with $n = 151$ – 448 single cell measurements). The Ca_i^{2+} release activated by propranolol had comparable time constants in both cancer cell lines. (B) Superimposed traces for individual cells of the propranolol-induced Ca_i^{2+} release in PC3 and MCF7 cell lines; arrowheads indicate the time of propranolol addition to the cells.

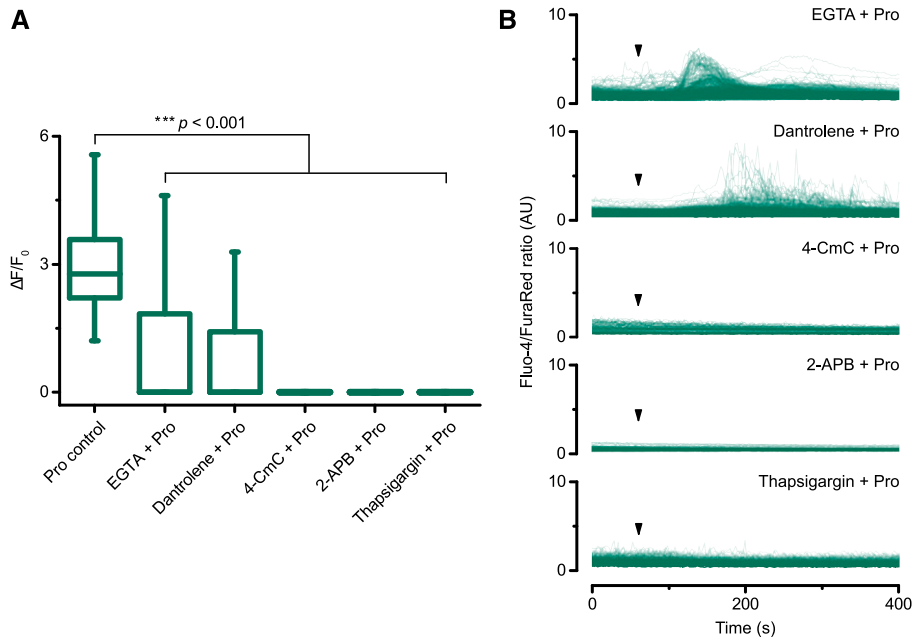


Fig. 6. Calcium-induced calcium release (CICR) as a putative mechanism for the propranolol-induced Ca_i^{2+} release. PC3 cells were treated with 50 μM propranolol (Pro; added as a bolus) and different chelators or inhibitors of CICR pathways; the mobilization of free Ca_i^{2+} release was monitored by time-lapse confocal microscopy. (A) Amplitude of the propranolol-induced Ca_i^{2+} release, calculated as fold increase in the fluorescence intensity of the calcium waveform ($\Delta F/F_0$). Box plots (L–R): 1) control cells treated with propranolol only, 2) EGTA 5 mM (chelation of extracellular calcium), 3) dantrolene 10 μM (inhibition of the ER RyR channels), 4) 4-CmC 1 mM (depletion of ER stores via activation of RyR channels), 5) 2-APB 50 μM (inhibition of the ER IP₃ receptor channels), and 6) thapsigargin 5 μM (exhaustion of ER stores via inhibition of SERCA pump). Blocking CICR pathways significantly inhibited the propranolol-induced Ca_i^{2+} release (Mann-Whitney *U* Test, $***P < 0.001$; $n = 3$ –8 imaging experiments and $n = 186$ –575 single-cell measurements per condition). (B) Superimposed traces for individual cells of the propranolol-induced Ca_i^{2+} release after EGTA, dantrolene, 4-CmC, 2-APB, or thapsigargin; the time of propranolol addition is indicated by arrowheads. A representative trace for propranolol-induced Ca_i^{2+} release can be found in Fig. 2A.

TRP channels (~100 channels, calculated based on the conductance of control cells' inward currents; Petrou et al., 2017) that may be responsible for the calcium influx required

for the activation of Ca_i^{2+} stores, and this may also be the case for propranolol. It is also plausible that there may be independent inhibition of whole-cell currents (based on the multiple

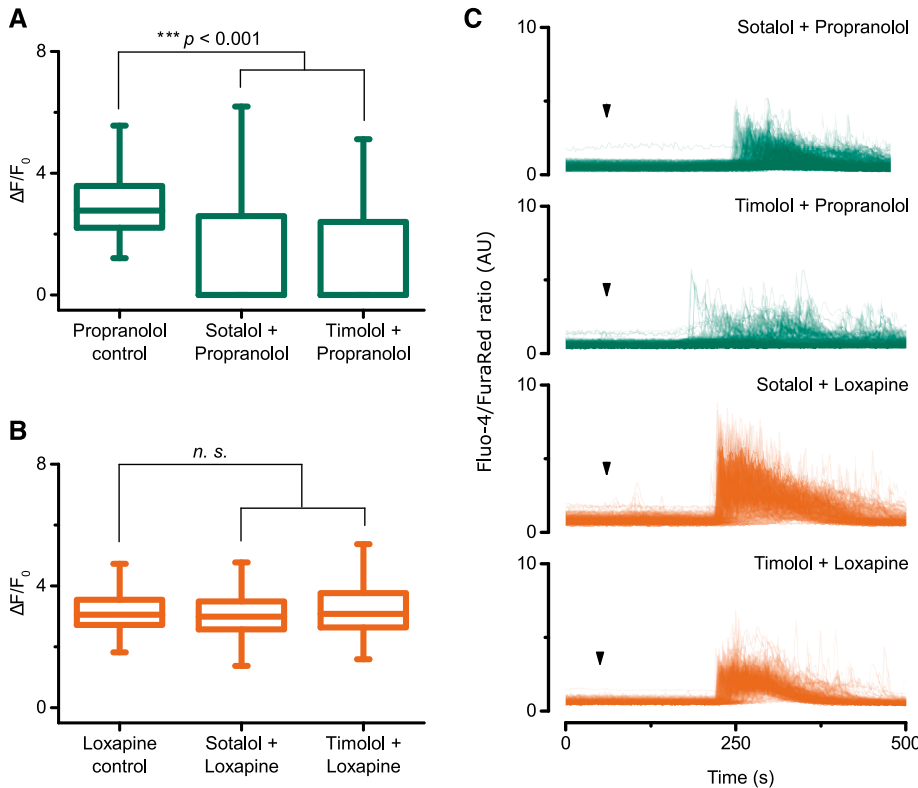


Fig. 7. Preincubation with nonselective β -blockers precludes normal propranolol-induced Ca_i^{2+} release. (A) PC3 cells were loaded with the calcium indicators Fluo-4 and FuraRed, as in previous experiments. Cells were pretreated with 250 μM of either sotalol or timolol (added as a bolus), nonselective β -blockers used to block the β -ARs. After 2 minutes of incubation, 50 μM propranolol was added to the cells, and intracellular calcium levels were monitored over time. Sotalol and timolol significantly inhibited the propranolol-induced Ca_i^{2+} release. (B) Control experiments were performed likewise using loxapine, a drug from a different pharmacologic class (i.e., dibenzoxazepine) that is known to activate Ca_i^{2+} release in these cells (Petrou et al., 2017). The loxapine-induced Ca_i^{2+} release was not affected by the blockade of β -ARs with sotalol or timolol ($n = 3$ –5 experiments and $n = 191$ –441 individual cells per condition; Mann-Whitney *U* Test, $***P < 0.001$, n.s., nonsignificant). (C) Superimposed traces for individual cells of the propranolol- and loxapine-induced Ca_i^{2+} release after sotalol and timolol incubation; the time of propranolol or loxapine addition is indicated by arrowheads. Representative Ca_i^{2+} release traces are shown in Fig. 2A for propranolol and Fig. 4 insert for loxapine.

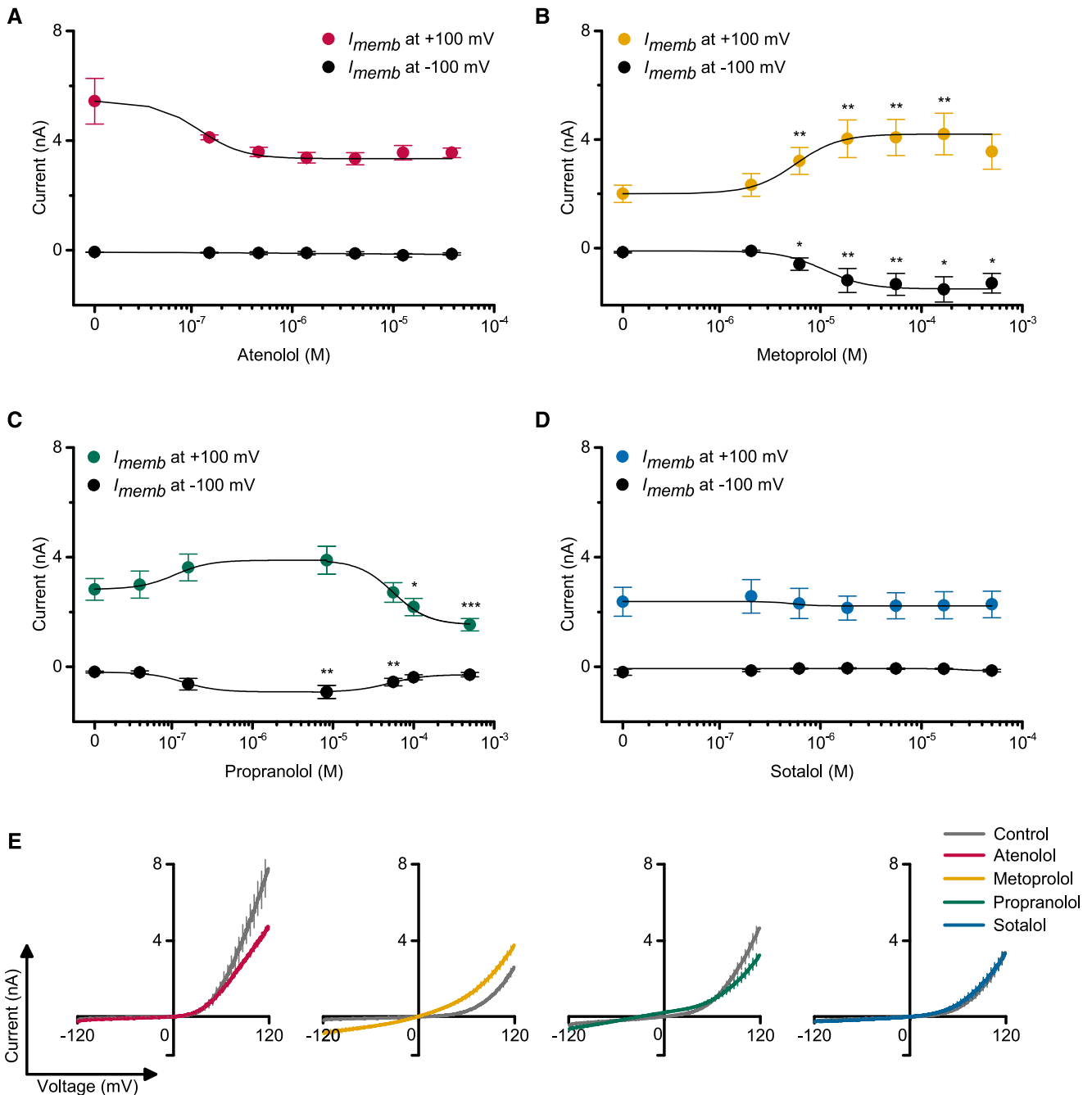


Fig. 8. β -Blocker regulation of endogenous whole-cell currents in PC3 cells. Single cells were recorded under whole-cell configuration using the QPatch automated cell patch-clamp system, which implements microfluidic control of drug delivery. (A–D) Endogenous whole-cell currents were measured by adding an increasing concentration (0–500 μ M, with solution changes within 500 milliseconds) of each β -blocker to the same cell clamped at a V_h of -10 mV and voltage ramped from -120 to $+120$ mV every 3 seconds. Currents at $+100$ and -100 mV were measured with or without β -blockers at six different concentrations and are shown as mean \pm S.E.M. ($n = 5$ –19 single cells per β -blocker). Changes in cell currents with respect to control (0 μ M) were analyzed using the Wilcoxon test; only significant changes are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (A) Atenolol, (B) metoprolol, (C) propranolol, and (D) sotalol. (E) Representative current-voltage (I–V) curves of control (gray lines) and with β -blockers (colored lines): atenolol at 38 μ M (magenta), metoprolol at 56 μ M (yellow), propranolol at 100 μ M (green), and sotalol at 50 μ M (blue) ($n = 5$ –30 cells; mean \pm S.E.M.).

interactions of propranolol with sodium and potassium ion channels; Supplemental Table 2), as well as an interdependent CICR-mediated mechanism of Ca_i^{2+} release. Such cellular functions for a commonly used antiarrhythmic agent may have implications on the pharmacology of this drug.

The addition of neither sotalol, which did not alter the endogenous whole-cell currents, nor metoprolol, which conversely

activated these currents, led to no increase in Ca_i^{2+} release. We speculate that atenolol, which does inhibit the endogenous currents but also does not activate Ca_i^{2+} release, may not be able to activate the downstream IP_3 pathway. The electrophysiological properties described here represent only a beginning of what appears to be intricate and complicated mechanisms regulating intracellular signals transduced by these drugs. Our results give

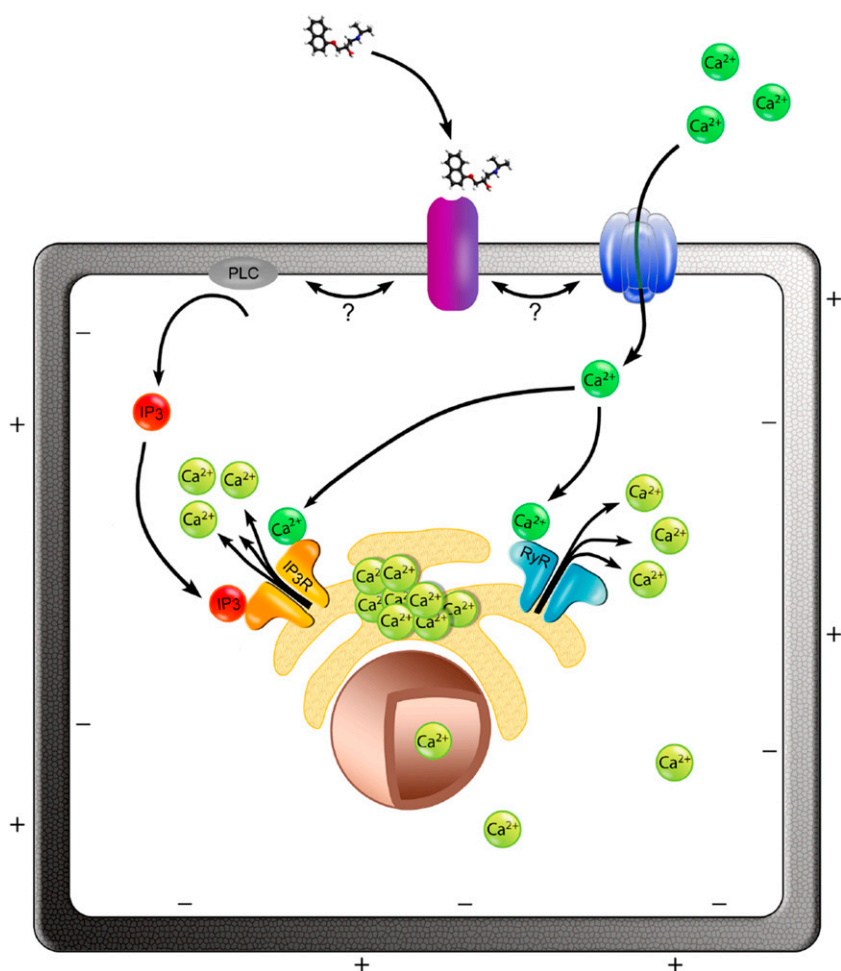


Fig. 9. Proposed model for the activation of Ca_i^{2+} release by propranolol via a CICR-facilitated mechanism. We propose that the binding of propranolol to its receptor in the cell membrane (purple) triggers the downstream activation of Ca_i^{2+} release from cellular stores (e.g., the ER depicted in the model). Extracellular calcium (dark green) contributes to the propranolol-induced Ca_i^{2+} release; small extracellular calcium influx may enter the cell via a calcium channel or a permeable cation channel (dark blue), which is activated by propranolol-receptor binding. Extracellular calcium promotes the release of Ca_i^{2+} from ER via RyRs (light blue). Additionally, propranolol-receptor binding results in the production of IP_3 (red) through the phospholipase C (PLC; gray), and this initiates a Ca_i^{2+} efflux via the IP_3 receptors (IP_3R ; orange) present in the ER surface. Once released, the free cytosolic calcium (light green) can enter the nucleus depolarizing the nuclear envelope.

an interesting first insight into how β -blockers might alter the electrical properties of nonexcitable cells, although their effects are complex and require further investigation.

Excitable and nonexcitable cells are thought to have different cell electrical properties but similar intracellular calcium signaling mechanisms (Putney, 1993). The pharmacology of β -blockers is often linked to the regulation of intracellular calcium, most notably in cardiac cells (Weiss et al., 2013). Our observation that β -blockers regulate Ca_i^{2+} release differently may reveal new mechanistic aspects of the action of these compounds. Atenolol and metoprolol, for example, are used mostly as antianginal and antihypertensive drugs; propranolol has a wider spectrum of applications besides its cardiovascular use (Joint Formulary Committee, 2017), such as the treatment of essential tremor (Zesiewicz et al., 2002) or anxiety (Steenen et al., 2016). Furthermore, if activation of Ca_i^{2+} stores by propranolol is initiated via the β_2 -AR (see later), this may imply that other tissues expressing this receptor, such as the lungs or blood vessels (Daly and McGrath, 2011), may undergo Ca_i^{2+} release events similar to those described in the cell lines used in this study.

Based on the data presented in our study, we propose a model of action for propranolol acting on intracellular calcium stores via a CICR-facilitated mechanism. Extracellular calcium contributes to the opening of the stores and the release of calcium through RyRs, and IP_3 acts as an intracellular transducer for the activation of Ca_i^{2+} release from the ER (Fig. 9).

Our observation that the β_2 -AR is the main subtype in PC3 cells at the gene expression level (Supplemental Table 3), as has been reported in human prostate tissue (Goepel et al., 1997; Suzuki et al., 2016) and other prostate cancer cell lines (Nagmani et al., 2003; Kasbohm et al., 2005), suggests that propranolol may be exerting its function via this receptor. This suggestion is supported by the observation that blockade of β -ARs with the nonselective β -blockers sotalol and timolol (Baker, 2005) inhibited the propranolol-induced Ca_i^{2+} release (Fig. 7), although this could also be attributed to nonselective cellular targets common to sotalol, timolol, and propranolol (Supplemental Table 2). MCF7 cells also express β -ARs (Supplemental Table 3; Shi et al., 2011; Işeri et al., 2014); even if the expression of the β -ARs is not as high in MCF7 cells as in other breast cancer cell lines (Vandewalle et al., 1990), radioligand binding assays have estimated that there are ~80,000 binding sites per cell in MCF7 cells (Gargiulo et al., 2014).

In the model we propose (Fig. 9), binding of propranolol to its receptor may activate extracellular calcium entry, which can trigger store-operated calcium release via CICR. Routes for calcium entry can include specific calcium channels as well as nonspecific cation channels that have significant calcium permeability, such as members of the TRP family (Clapham et al., 2001), some of which are known to be expressed in cancer cells (Bödding, 2007), including prostate cancer (Wissenbach et al., 2004; Bidaux et al., 2007; Prevarskaya et al., 2007). The focus of our future studies will be to

investigate the receptors, ion channels, and downstream pathways responsible for the propranolol-induced Ca_i^{2+} release described here and examine the effects in the transcriptional profile of cancer cells.

Propranolol is known to inhibit cell migration in several *in vitro* and *in vivo* models of cancer, including breast (Campbell et al., 2012; Işeri et al., 2014; Pon et al., 2016), colon (Masur et al., 2001; Işeri et al., 2014), angiosarcoma (Stiles et al., 2013), and prostate cancer (Palm et al., 2006). Free Ca_i^{2+} is an important regulator of tumor metastasis, and several Ca_i^{2+} -dependent mechanisms contribute to malignant cell migration (Prevarskaya et al., 2011). Propranolol has shown greater antimigratory effects than other β -blockers, such as atenolol (Masur et al., 2001; Işeri et al., 2014). In cancer cell lines, including PC3 cells, propranolol (between 100 and 200 μM) was shown to inhibit proliferation and induce apoptosis (Zhang et al., 2010; Brohé et al., 2015; Coelho et al., 2015; Wrobel and Le Gal, 2015; Chin et al., 2016; Wei et al., 2016; reviewed in Pantziarka et al., 2016). The concentration at which propranolol exerted these antiproliferative and proapoptotic effects is within the range at which we observe propranolol-induced Ca_i^{2+} release in PC3 and MCF7 cells. It is also notable that in the same studies (Zhang et al., 2010; Coelho et al., 2015; Wrobel and Le Gal, 2015; Chin et al., 2016; Wei et al., 2016) neither atenolol nor metoprolol inhibited cell proliferation or induced apoptosis.

Our results point to a novel action of propranolol and its potential as a regulator of the magnitude and duration of Ca_i^{2+} release *in vitro*. Our finding that propranolol mobilizes free Ca_i^{2+} , which distinguishes this drug from other commonly used β -blockers, opens new possibilities into how propranolol may contribute to the inhibition of malignant cell migration and proliferation, whereas other β -blockers that do not activate Ca_i^{2+} release may not exert the same effect. This mechanism may thus be relevant patients who are treated with these drugs (Baker et al., 2011; Pantziarka et al., 2016).

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Authorship Contributions

Participated in research design: Ahmed, Reyes-Corral, Sørensen.
Conducted experiments: Reyes-Corral, Sørensen.
Contributed to new reagents or analytical tools: Thrasivoulou, Dasgupta, Ashmore.
Performed data analysis: Reyes-Corral, Sørensen, Ashmore, Ahmed.
Wrote or contributed to the writing of the manuscript: Reyes-Corral, Ashmore, Ahmed.

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