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Tiotropium bromide, a long acting muscarinic receptor antagonist triggers intracellular calcium signalling in the heart

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Abstract

Background and Purpose:

Tiotropium bromide (TB) is a long acting muscarinic receptor antagonist used to manage chronic obstructive pulmonary disease (COPD). Recent meta-analyses suggest an increased risk of cardiovascular events with TB. Ca^{2+}/calmodulin dependent kinase II (CaMKII) and L-type Ca^{2+} channels regulate Ca^{2+} concentrations allowing management of Ca^{2+} across membranes, however pathological increases are initially slow and progressive but once the cytosolic concentration rises >1-3 μM from ~100 nM, calcium overload occurs and can lead to cell death. Ipratropium bromide, a short acting muscarinic receptor antagonist has previously been found to induce Ca^{2+} mediated eryptosis. The aim of this study was to investigate the role of Ca^{2+} in Tiotropium bromide mediated cardiotoxicity.

Experimental approach:

Isolated Sprague-Dawley rat hearts were perfused with TB (10 – 0.1 nM) ± KN-93 (400 nM) or nifedipine (1 nM). Hearts were stained to determine infarct size (%) using triphenyltetrazolium chloride (TTC), or snap frozen to determine p-CaMKII (Thr_{286}) expression. Cardiomyocytes were isolated using a modified Langendorff perfusion and enzymatic dissociation before preparation for Fluo 3-AM staining and flow cytometric analysis.

Key results:

TB increased infarct size compared with controls by 6.91-8.41%, with no effect on haemodynamic function. KN-93/nifedipine with TB showed a 5.90/7.38% decrease in infarct size compared to TB alone, the combined use of KN-93 with TB also showed a significant increase in left ventricular developed pressure whilst nifedipine with TB showed a significant decrease in coronary flow. TB showed a 42.73% increase in p-CaMKII (Thr_{286}) versus control, and increased Ca^{2+} fluorescence by 30.63% in cardiomyocytes.

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Conclusions and implications:

To our knowledge, this is the first pre-clinical study to show that Tiotropium bromide induces Ca^{2+} signalling via CaMKII and L-type Ca^{2+} channels to result in cell damage. This has significant clinical impact due to long term use of TB in COPD patients, and warrants assessment of cardiac drug safety.

Keywords: Long acting muscarinic receptor antagonist; Cardiotoxicity; Tiotropium bromide; Calcium; Ca^{2+}/Calmodulin kinase II (CaMKII)

Abbreviations

BPM	Beats per minute
CICR	Ca^{2+} induced Ca^{2+} release
CaMKII	Ca^{2+}/calmodulin dependent protein kinase II
KRH	Calcium free Krebs buffer
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
LSD	Fisher's least significant difference
IP_3	Inositol 1,4,5 triphosphate
IP_3R	IP_3 receptor
KH	Krebs-Henseleit
LDH	Lactate dehydrogenase
LVDP	Left ventricular developed pressure
SEM	Mean standard error
NCX	Na^+/Ca^{2+} exchanger
-ve	Negative
Nif	Nifedipine
NO	Nitric Oxide
PIP2	Phosphatidylinositol 4,5 bisphosphate
PLC	Phospholipase C
PVDF	Polyvinylidene fluoride
+ve	Positive
SR	Sarcoplasmic reticulum
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}/ATPase
TB	Tiotropium bromide
TTC	Triphenyltetrazolium chloride

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Introduction

The importance of cellular calcium regulation is demonstrated in several processes such as excitability, cell motility and gene transcription; numerous sensor and adaptor proteins respond to changes in calcium concentrations and initiate cellular responses (Clapham, 2007). Disturbances in calcium regulation can negatively affect cellular function and ultimately lead to cell death, best exemplified with compartmental dependence on calcium homeostasis, and its role in initiating cell death signalling (Orrenius et al., 2003).

Calcium signalling is instrumental in the functioning heart and plays roles in contractility and gene transcription (Ronkainen et al., 2011). In cardiomyocytes, systole occurs via L-type Ca^{2+} channels, triggering Ca^{2+} induced Ca^{2+} release (CICR) via the sarcoplasmic reticulum (Bootman, 2012). Relaxation of cardiomyocytes occurs following Ca^{2+} removal via the Na^{+}/Ca^{2+} exchanger (NCX), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Fearnley et al., 2011; Murphy & Steenbergen, 2008). The importance of intracellular calcium regulation is testified by the number of Ca^{2+} handling proteins involved in this process (e.g. calmodulin, calpains and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII)).

Whilst calcium is necessary for cellular function, detrimental calcium influx can be linked to the dysfunction of several key channels. For example, sarcolemmal L-type calcium channels and SERCA dysfunction lead to hypercontracture and mitochondrial damage, resulting in cell death (Turer & Hill, 2010). In this context the ‘calcium hypothesis’ suggests that the cellular ability to regulate calcium is impaired following ischaemia, resulting in accumulation of toxic intracellular concentrations of Ca^{2+} through activation of the NCX, leading to mitochondrial dysfunction and apoptosis mediated cell death (Moens et al., 2005).

Anti-muscarinics such as the long acting muscarinic receptor antagonist, Tiotropium bromide are used in the treatment of chronic obstructive pulmonary disease (COPD). However, a meta-analysis showed that cardiovascular risk, myocardial infarction and stroke were at

increased risk amongst COPD patients using anti-muscarinics (Singh et al., 2008). A recent clinical study also correlated the use of anti-muscarinics with adverse cardiovascular outcomes in patient trials (Liou et al., 2018), therefore indicating adverse cardiovascular effects with drug use. In particular, Ipratropium bromide, a short acting anti-muscarinic has been shown to exacerbate myocardial ischaemia/reperfusion injury in an *in vitro* whole heart model (Harvey et al., 2014) and initiate calcium mediated suicidal cell death of erythrocytes (Shaik et al., 2012), indicating the occurrence of intercellular signalling otherwise not ascribed to antagonists.

In this study, we examine the role of Tiotropium bromide in Ca^{2+} mediated cardiotoxicity. Using whole heart rodent models, isolated cardiomyocytes and pharmacological inhibition of L-type Ca^{2+} channels and CaMKII, we investigate the effect of Tiotropium bromide on physiological calcium regulation and cellular signalling in the heart.

Methods

Animals and ethics

Prior to experimentation, all studies were approved by the Coventry University Ethics committee (UK). Adult 3-month old male Sprague-Dawley rats (300g ± 50g body weight – Charles River, UK) were kept in humane conditions and fed a standard laboratory diet. Procedures were in accordance with the Guidelines on the Operation of the Animals (Scientific Procedures) Act 1986. Whole hearts were used for Langendorff models to record haemodynamic parameters and evaluate infarct development, tissue collection (Western blotting) and cardiomyocyte cell isolations. Group sizes were determined based on pilot data (data not shown) and as described by Harvey et al. (2014), all groups were designed to be equal, any loss in numbers were from experimental deviations.

Whole heart Langendorff model and haemodynamic function

Animals were euthanised via cervical dislocation in accordance with the Schedule I Home Office procedure. Intact hearts were excised and immediately placed in ice cold Krebs-Henseleit (KH - 118.5 mM NaCl, 25 mM $NaHCO_3$, 12 mM Glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$ and 1.7 mM $CaCl_2.2H_2O$) buffer before mounting onto the Langendorff apparatus, and retrogradely perfused with KH buffer (37^{o}C, pH 7.4) saturated with 95% O_2 and 5% CO_2. Tiotropium bromide (10 nM – 0.1 nM) was examined at clinically relevant concentrations, with 1 nM subsequently used for all other studies along with KN-93 (400 nM) and nifedipine (1 nM). Experimental protocols were as described by Gharanei et al. (2013). Haemodynamic parameters (coronary flow, heart rate and left ventricular developed pressure) were recorded at 5 minute intervals for the first 55 minutes and 15 minute intervals thereafter, using a physiological pressure transducer connected to a PowerLab (ADI, UK) and LabChart® software (v7). Controls received only Krebs-Henseleit (KH) buffer for the duration of the protocol (175 mins) with no drug intervention or protocol differences, all subsequent groups defined as 'control' or 'normoxia' followed this protocol [Supplementary File, S1]. For all drug groups (Tiotropium bromide, KN-93 and nifedipine), drug administration was initiated following 20 minutes of stabilisation and remained for the remainder of the protocol (155 minutes). Hearts were weighed at the end of the experimental protocol and stored at -20^{o}C, before triphenyltetrazolium chloride (TTC) staining.

Infarct size

The TTC staining procedure was as previously described by Bell et al. (Bell, Mocanu, & Yellon, 2011). Frozen Langendorff hearts (-20^{o}C) were sliced to obtain approximately 2 mm thick transverse slices and incubated in 1% TTC phosphate buffer (pH 7.4) at 37^{o}C for 10 minutes. Heart slices were then removed from the TTC buffer and placed in 10% formalin solution for a minimum of 4 hours prior to delineate viable and non-viable tissue. Viable tissue appeared red (tetrazolium positive) whilst infarcted areas appeared pale (tetrazolium negative) [Supplementary File, S2]. Areas of infarction were measured using ImageJ (NIH, Wisconsin, USA) by computerised planimetry to obtain an area percentage (infarct %).

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Adult ventricular cardiomyocyte isolation

Hearts were obtained as described above, and mounted onto a modified Langendorff set up perfused with a calcium free Krebs buffer (KRH - 119.9 mM NaCl, 5.4 mM KCl, 0.49 mM $MgSO_4$, 10 mM Glucose, 19.98 mM Taurine, 5 mM Sodium Pyruvate, 5.06 mM Na_2HPO_4, 11.76 mM KH_2PO_4 oxygenated with 95% O_2 and 5% CO_2, pH 7.4, 37^{o}C) via a mechanical pump set at a constant flow of 10 mls/min. Hearts were perfused for 3 minutes to ensure cessation of cardiac function before 5-7 minutes of perfusion with modified digestion buffer (KRH, 0.046% Gibco® Collagenase Type II, 34 µM $CaCl_2$); during digestion, the coronary perfusate was collected and recycled throughout the 5-7 minute time period.

Following enzymatic digestion, ventricular tissue was minced and aspirated for 5 minutes in digestion buffer for complete enzymatic dissociation, before filtration through nylon mesh. The filtrate was centrifuged (1200 RPM, 2 minutes) and the pellet was resuspended in restoration buffer composed of modified Krebs buffer (KRH, 5 mM Creatine, 50 µM $CaCl_2$, 1% BSA, 1% Pen/Strep) and maintained at 37^{o}C; 3.4 µl of 1 M $CaCl_2$ was added gradually to increase the calcium concentration to 1.13 M.

Western blotting for phosphorylated CaMKII$_{Thr286}$

Left ventricular tissue was excised following Langendorff perfusion, and rapidly frozen in liquid nitrogen and stored at -80^{o}C. Prior to use, frozen tissue were homogenised with lysis buffer (100 mM NaCl, 10 mM Tris base - pH 8.0, 1 mM EDTA - pH 8.0, 2 mM sodium pyrophosphate, 2 mM NaF, 2 mM β-glycerophosphate, Sigma*FAST*™ protease inhibitor cocktail tablets – 1 tablet/100ml and PhosStop™ - 1 tablet/10ml) and centrifuged (11000 RPM, 10 minutes, 4^{o}C) to obtain the supernatant. Protein content was calculated using the Pierce™ BCA assay kit (Thermo Fisher Scientific, UK). Samples were diluted to obtain a protein concentration of 50 µg using Laemmli buffer (250 mM Tris-HCl – pH 6.8, 10% glycerol, 0.006% bromophenol blue, 4% SDS, β-mercaptoethanol – pH 6.8) and incubated at 100^{o}C for 5 minutes. Samples were loaded and run using a Power-Pac 3000 (130-150V, 60-

75 minutes; Bio-Rad, UK), a semi-dry transfer system was used for protein transfer (PVDF Trans-Blot® Turbo™, mixed molecular weight transfer, 7 minutes; Bio-Rad, UK). PVDF blots were incubated in blocking buffer (5% w/v milk powder in Tris-buffered saline with 1% Tween 20 - TBS/T, pH 7.4) for 60 minutes on an orbital shaker prior to overnight primary antibody incubation as instructed by the manufacturer (5% w/v bovine serum albumin (BSA) in TBS/T, 4^{o}C). Membranes were washed with TBS/T and incubated with secondary antibody in blocking solution (5% w/v milk powder in TBS/T, 1 hr) on an orbital shaker before visualisation using SuperSignal West Femto (Thermo Fisher Scientific, UK) and a ChemiDoc transluminator (Bio-Rad, UK) with ImageLab®. Membranes were auto-exposed to detect dense bands corresponding to the protein of interest. Images were captured and analysed by visual densitometry using ImageJ (NIH, USA).

Calcium release measurement

Fluo 3-AM (Life Technologies, UK) stock solution was reconstituted using DMSO (500 µM) and added to cardiomyocytes following incubation with Tiotropium bromide (1 nM) ± KN-93 (400 nM), ionomycin (1 µM) as a positive control and amiloride (100 mM) as a negative control, at a final concentration of 5 µM and incubated for 25 minutes, away from light. Cells were centrifuged (300 RPM, 2 minutes) and resuspended in fresh restoration buffer before incubation for de-esterification (10 minutes). Cells were immediately analysed using the FL-1 channel of the flow cytometer (BD FACSCalibur, UK) at 488 nm excitation, set to count 10,000 cells. All data points were normalised to the control group to account for background fluorescence.

Statistical analysis

Data presented in this study are expressed as the mean ± standard error of the mean (SEM). GraphPad Prism 7® and SPSS® v24 (IBM) software were used to statistically analyse the data. For haemodynamics and infarct data, one-way ANOVA with Tukey's test

were used; cardiomyocyte data were analysed using ANOVA with Fisher's Least Significant Difference (LSD) post-hoc test. A p-value of $p<0.05$ was considered statistically significant.

Materials

Tiotropium bromide (Sequoia Research Products Ltd, UK) was dissolved in Dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) before experimental use and diluted in Krebs Henseleit buffer for subsequent dilutions. 2,3,5-Triphenyltetrazolium Chloride (TTC) (Sigma Aldrich, UK) was used at a final concentration of 2%. KN-93 (CaMKII inhibitor), Nifedipine (L-type Ca^{2+} channel blocker), Ionomycin (Ca^{2+} ionophore) and Amlodipine (L-type Ca^{2+} channel blocker) were purchased from Tocris (Bristol, UK) and dissolved in DMSO and stored at -20^{o}C.

Anti-CaMKII, p-CaMKII $_{(Thr286)}$ antibody and secondary HRP-linked anti-rabbit IgG were purchased from Cell Signaling, UK (Cell Signaling Technology Cat# 4436 and 3361). Mini-PROTEAN® TGX™ pre-cast gels, the Mini-PROTEAN tetra cell, Trans-blot® Turbo™, and PVDF membranes were purchased from Bio-Rad Ltd. (UK).

Results

Effect of Tiotropium bromide on whole heart haemodynamic function and infarct size.

Tiotropium bromide (TB) 0.1 nM showed an increase in coronary flow compared to TB 10 nM at 70 minutes (109.34 ± 18.18% (TB 0.1 nM) vs. 73.57 ± 6.61% (TB 10nM), fig. 1a). There was no significant difference observed in coronary flow with all 3 concentrations when compared to the control group. Differences observed in LVDP were significant towards the end of the experimental protocol, at 160 mins. Both the 10 nM and 0.1 nM concentrations of Tiotropium bromide showed increased LVDP with respect to the control at 160 mins (100.11 ± 5.43% (10 nM) and 104.47 ± 1.19% (0.1 nM) vs. 83.19 ± 2.89% (control), fig. 1b). The LVDP at 160 mins was increased with respect to the 1 nM concentration of Tiotropium bromide (100.11 ± 5.43% (TB 10 nM) and 104.47 ± 1.19% (TB 0.1 nM) vs. 76.54 ± 6.11%

(TB 1nM), fig. 1b). There was no difference in heart rate amongst the treatment groups (fig. 1c).

Infarct sizes were calculated as a percentage of heart tissue found to be TTC negative. Administration of Tiotropium bromide (10 – 0.1 nM) saw increased infarct size compared to the control (fig.2). Tiotropium bromide (1 nM) showed the most increase in infarct size with respect to the control (18.69 ± 1.79% vs. 10.28 ± 1.74%, fig. 2). Both the 10 nM and 0.1 nM concentrations also showed increased infarct size of comparable magnitude, compared to the control (17.55 ± 0.98% (TB 10 nM) and 17.19 ± 0.37% (TB 0.1 nM) vs. 10.27 ± 1.94% (control), fig. 2).

Effect of KN-93 administration on whole heart haemodynamic function and infarct size.

Administration of the CaMKII inhibitor, KN-93 (400 nM) alone or co-administered with Tiotropium bromide (1 nM) showed no significant effect on the coronary flow of the heart (fig. 3a) and the heart rate (fig. 3c), however, significant differences were observed in LVDP. KN-93, when co-administered with Tiotropium bromide, showed a significant increase in LVDP at 50 mins with respect to KN-93 alone (131.35 ± 12.62% (TB 1nM + KN-93 400nM) vs. 91.75 ± 6.36% (KN-93 400nM), $p<0.05$, fig. 3b). This increase in LVDP following KN-93 and Tiotropium bromide co-administration was further observed at 160 mins with respect to all other treatment groups (103.47 ± 7.28% (TB 1nM + KN-93 400nM) vs. 83.19 ± 2.89% (control, $p<0.05$), 76.45 ± 6.11% (TB 1nM) and 77.14 ± 3.01% (KN-93 400nM), $p<0.01$, fig. 3b).

KN-93 (400 nM) showed no significant difference in infarct size compared to the control, however this was significantly lower than that observed with Tiotropium bromide (1 nM) (9.50 ± 0.90% (KN-93 400nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.01$, fig. 4). This significance was sustained upon co-administration of KN-93 and Tiotropium bromide with respect to Tiotropium bromide alone, which showed reduced infarct development upon co-administration (12.81 ± 2.81% (TB 1nM + KN-93 400nM) vs. 18.69 ± 1.79% (TB 1nM), $p<0.05$, fig. 4).

Effect of Nifedipine on whole heart haemodynamic function and infarct size.

The use of the L-type Ca^{2+} channel inhibitor, nifedipine (1 nM) in a whole heart Langendorff model showed a significant decrease in coronary flow when co-administered with Tiotropium bromide (1 nM), with respect to nifedipine (1 nM) alone both at 50 mins and at 70 mins (50 minutes: 80.44 ± 6.02% (TB 1nM + Nif 1nM) vs. 99.98 ± 7.30% (Nif 1nM), $p<0.05$; 70 minutes: 71.31 ± 8.83% (TB 1nM + Nif 1nM) vs. 98.98 ± 3.00% (Nif 1nM), $p<0.01$, fig. 5a). Administration of nifedipine did not show a significant difference in LVDP (fig. 5b) or heart rate (fig. 5c).

Much like KN-93, nifedipine (1 nM) did not see a significant difference in infarct size compared to the control; however, nifedipine showed a significant decrease in infarct size when compared to Tiotropium bromide (1 nM) (6.32 ± 0.77% (Nif 1 nM) vs. 18.69 ± 1.79% (TB 1 nM), $p<0.001$, fig. 6). This significant decrease was also observed when Nifedipine was co-administered with Tiotropium bromide (11.32 ± 1.66% (TB 1 nM + Nif 1 nM) vs. 18.69 ± 1.79% (TB 1 nM), $p<0.01$, fig. 6).

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Effect of Tiotropium bromide on CaMKII phosphorylation in the presence and absence of KN-93.

CaMKII activation was assessed using western blotting for phosphorylated CaMKII $_{(Thr286)}$ in cardiac tissue, to establish the role of CaMKII in Tiotropium bromide (1 nM) mediated cardiotoxicity, with or without the CaMKII inhibitor, KN-93 (400 nM). Tiotropium bromide showed a significant increase in CaMKII phosphorylation with respect to the control (86.46 ± 8.43% (Tiotropium bromide 1 nM) vs. 43.73 ± 5.71% (control), fig. 7). The administration of KN-93 did not show any significant difference in CaMKII phosphorylation with respect to the control, however CaMKII phosphorylation with Tiotropium bromide was significantly greater than KN-93 alone (51.84 ± 8.11% (KN-93 400 nM) vs. 86.46 ± 8.43% (Tiotropium bromide 1 nM), fig. 7). The increase in CaMKII phosphorylation observed with Tiotropium bromide was significantly attenuated when co-administered with KN-93, with respect to Tiotropium bromide alone (59.58 ± 8.94% (TB 1nM + KN-93 400nM) vs. 86.46 ± 8.43% (Tiotropium bromide 1nM), fig. 7).

Effect of Tiotropium bromide on ventricular cardiomyocyte calcium release in the presence and absence of KN-93.

Tiotropium bromide showed a significant increase in Fluo 3-AM fluorescence with respect to the control (130.63 ± 1.65% (TB 1nM) vs. 100 ± 0.0% (Control), $p<0.05$, fig. 8). Conversely, KN-93 did not show any significant difference in Fluo 3-AM fluorescence with respect to the control and Tiotropium bromide groups. The co-administration of KN-93 with Tiotropium bromide and Tiotropium bromide alone showed a significant increase in Fluo 3-AM with respect to the negative control, Amiloride (93.92 ± 10.01% (Amiloride 100 mM) vs. 130.63 ± 1.65% (TB 1 nM) and 124.34 ± 22.30% (TB 1 nM + KN-93 400 nM), $p<0.05$, fig. 8). Ionomycin showed a significant increase in Fluo 3-AM fluorescence with respect to the

control and negative control, amiloride (135.69 ± 9.17% (Ionomycin 1 μM) vs. 100 ± 0.0% (Control) and 93.92 ± 10.01% (Amiloride 100 mM), $p<0.01$, fig. 8).

Discussion

The present study demonstrated that Tiotropium bromide altered calcium signalling, resulting in cell damage within the heart. Interestingly, the damage inflicted as a result of Tiotropium bromide administration had no effect on normal haemodynamic function. Additionally, this effect was attenuated through inhibition of L-type Ca^{2+} channels and CaMKII.

Tiotropium bromide is a long acting muscarinic receptor antagonist with higher affinity for the M_3 receptor subtype than the M_2, functioning to inhibit cholinergic mediated bronchoconstriction within airway smooth muscle cells (Barnes et al., 2003). The M_2 receptor subtype is known to be the predominant form in the heart, however M_3 receptors are also present in the heart, and the function of these receptors has largely been attributed to pathological conditions such as ischaemia/reperfusion injury (Wang et al., 2012). The presence of muscarinic receptors in the heart has meant that adverse cardiovascular effect has always been a cause of concern for anti-muscarinics. Previous research has focused on the adverse cardiovascular effects associated with the short acting muscarinic receptor antagonist, Ipratropium bromide.

However, recent studies have associated a link with increased cardiovascular events amongst COPD patients using anti-muscarinics such as Tiotropium bromide (Liou et al., 2018; Arana et al., 2018; Koehorst-ter Huurne et al., 2018; Zou et al., 2018). Studies have assessed the use of Tiotropium bromide in comparison to β-adrenergic agonists and combination therapies; these meta-analyses have suggested that new use of long acting anti-muscarinics may be subjected to a greater risk of cardiovascular adverse effects (Wang et al., 2018). Although there have been discrepancies in the magnitude of cardiac effects

associated with anti-muscarinics (Wise et al., 2013), these studies corroborate with previous meta-analyses which suggest that Tiotropium bromide may affect cardiovascular risk amongst COPD patients (Singh et al., 2008; Singh et al., 2011). Interestingly, the data shown in this study proposes Ca^{2+} involvement in Tiotropium bromide mediated disturbances within the naïve heart, without significantly disrupting haemodynamic function, but affecting cell death as observed through exacerbated infarct size. A pilot study to measure cell death in cardiomyocytes with Tiotropium bromide was conducted using Annexin-V/PI staining. An increasing trend in apoptotic cells with increasing concentrations, however the concentrations used in this study (10 – 0.1 nM) did not show a significant increase in apoptosis [Supplementary File, S3].

Ca^{2+} overload results in reversal of Na^{+}/Ca^{2+} exchanger (NCX) function leading to increased intracellular Ca^{2+} release (Garcia-Dorado et al., 2012; Krebs et al., 2015). L-type Ca^{2+} channels mediate cellular calcium entry during excitation-coupling (Murgia & Rizzuto, 2014), leading to calcium induced calcium release (CICR); cardiotoxic drugs have been shown to increase intracellular Ca^{2+} (Hahn et al., 2014). Nifedipine is an L-type Ca^{2+} inhibitor; studies in isolated hearts have found nifedipine to reduce lactase dehydrogenase (LDH) leakage and neutrophil accumulation following myocardial ischaemia/reperfusion injury (Huang et al., 2009). Nifedipine has negative inotropic and chronotropic effects but increases coronary flow at low concentrations (Kumar et al., 2012). In the present study, nifedipine significantly decreases infarct size, compared to Tiotropium bromide and co-administration abrogates Tiotropium bromide mediated infarct size. The protective effect of nifedipine on infarct size has been linked to nitric oxide (NO) production (Huang et al., 2009; Kitakaze et al., 2000). Co-administration of nifedipine with Tiotropium bromide also shows significant reduction in coronary flow. Muscarinic receptor activation has previously been associated with acetylcholine mediated vasoconstriction in rodent coronary vessels (Nasa et al., 1997), however the results of this study suggest that Tiotropium bromide induces vasoconstriction when administered with nifedipine despite the effect of nifedipine on NO. This suggests the

involvement of other Ca^{2+} mediators aside from L type Ca^{2+} channels in Tiotropium bromide mediated cardiotoxicity, as nifedipine administration dampens the effect of Tiotropium bromide on infarct size, but does not reverse it.

A marked increase in CaMKII activity and abnormal Ca^{2+} release is observed in heart failure and arrhythmias (Fischer et al., 2013; Sossalla et al., 2010). M_1, M_3 and M_5 receptors increase endoplasmic reticulum Ca^{2+} via phospholipase C (PLC), inositol 1,4,5 triphosphate (IP_3) and calmodulin-dependent mechanisms (Pronin et al., 2017). CaMKII in ischaemia/reperfusion and myocardial infarction promotes cell death through mPTP opening and phosphorylation of ryanodine receptors (Di Carlo et al., 2014; Joiner et al., 2012). KN-93 inhibition prevents calmodulin-CaMKII interaction and inhibits increases in sarcoplasmic reticulum (SR) Ca^{2+}, leading to decreased excitation coupling (Zhang et al., 2004). However, KN-93 does not inhibit the catalytic activity of auto-phosphorylated CaMKII (Pellicena and Schulman 2014). KN-93 is good for assessing the function of CaMKII, however due to its competitive nature with calmodulin, autonomous activity can't be inhibited, additionally, it also affects voltage gated K^+ and Ca^{2+} channels (Ledoux, Chartier and Leblanc 1999, Vest et al. 2007). This study suggests that Tiotropium bromide increases CaMKII phosphorylation, which is attenuated but not fully reversed by KN-93, therefore suggesting auto-phosphorylation of CaMKII. This study shows elevated Ca^{2+} in cardiomyocytes, through increased Fluo 3-AM fluorescence following Tiotropium bromide administration; this increase is attenuated with the use of KN-93 which suggests that CaMKII may be involved in Tiotropium bromide mediated Ca^{2+} release.

CaMKII inhibition in addition to Tiotropium bromide shows enhanced LVDP function, but no other functional changes. Studies have shown that CaMKII inhibition in failing hearts improves contractility (Vila-Petroff et al., 2007); the data from this study suggests that inhibition of CaMKII improves LVDP function following Tiotropium bromide. As well as improvement in LVDP, there is also a protective effect observed with CaMKII inhibition of Tiotropium bromide mediated infarct size. Constitutive activation of ryanodine receptors by

CaMKII is known to increase cardiac injury (Di Carlo et al., 2014) and inhibition of CaMKII has been shown to decrease infarct size (Salas et al., 2010; Vila-Petroff et al., 2007). This study suggests that CaMKII inhibition may have beneficial effects on Tiotropium bromide mediated cardiac damage in naïve hearts.

Few studies have evidenced pre-clinical data to assess the effect of muscarinic receptor antagonists on the heart. To our knowledge the only study which has observed the effect of anti-muscarinics in a cardiac model has shown Ipratropium bromide to exacerbate myocardial ischaemia/reperfusion injury via apoptotic mediators such as caspase-3 (Harvey et al., 2014). Tiotropium bromide has been described both as an inverse agonist (Kruse et al., 2012) and an antagonist (Cooke et al., 2015), our study suggests that Tiotropium bromide may exert an effect as an inverse agonist (figure 9), due to increased intracellular Ca^{2+} following Tiotropium bromide administration in cardiomyocytes, and increased infarct size compared to controls. M_3 receptor activation leads to $G\alpha_{q/1q}$-PLC mediated cleavage of phosphatidylinositol 4,5 bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG); IP_3 in particular leads to sarcoplasmic reticulum calcium release via its receptor (IP_3R) (Terrar et al., 2018). However further investigations are necessary to determine the role of Tiotropium bromide as an inverse agonist capable of eliciting a signalling effect in the heart.

This study identifies dissociation between cardiac functional parameters and potentially detrimental cellular signalling, therefore highlighting a need for improved assessment of cardiotoxicity. Vascular biomarkers have recently been introduced in identifying at-risk populations amongst COPD patients, including aortic pulse wave velocity and intima media thickening, which have shown COPD to be an independent risk factor for cardiovascular disease (Fisk et al., 2018). The importance of translatable pre-clinical research cannot be emphasised enough, and it is vital to ensure that the effects observed in animal models are representative of *in vivo* signalling processes. This study has shown the effects of Tiotropium bromide in naïve conditions, finding detectable changes in cardiac damage in a Ca^{2+} mediated manner.

This is the first pre-clinical study to show that Tiotropium bromide alters calcium signalling in the heart through modulation of intracellular calcium release and enhanced activation of the Ca^{2+}/Calmodulin dependent protein kinase II (CaMKII) in naïve hearts, without significantly affecting haemodynamic function.

The results of this study have considerable impact on clinical decision making, particularly regarding COPD patients who are at greater risk of cardiovascular complications. Whilst COPD itself is a risk factor for cardiovascular disease, the use of drugs, which may elevate this risk without significantly altering cardiovascular function, raises an urgent need to identify and screen for changes occurring at a cellular level, prior to major cardiovascular remodelling which may affect haemodynamic function.

In conclusion, this study provides evidence that intracellular Ca^{2+} signalling follows Tiotropium bromide administration in cardiac models. The pharmacological inhibition of CaMKII and L-type Ca^{2+} channels show protective effects on Tiotropium bromide mediated cardiac injury and therefore warrants further investigation of the cellular processes underlying these effects. It is necessary to implement changes to how COPD patients are assessed for adverse cardiovascular effects, in order to precipitate the cellular changes which may be occurring as a result of off-target drug effects.

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Author Contribution

SC contributed to the conception, design, drafted the manuscript, data analysis and interpretation of the work and critically revised the manuscript. AH and CJM contributed to conception, design, data analysis, interpretation of the work and critically revised the manuscript. DR critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work ensuring integrity and accuracy.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

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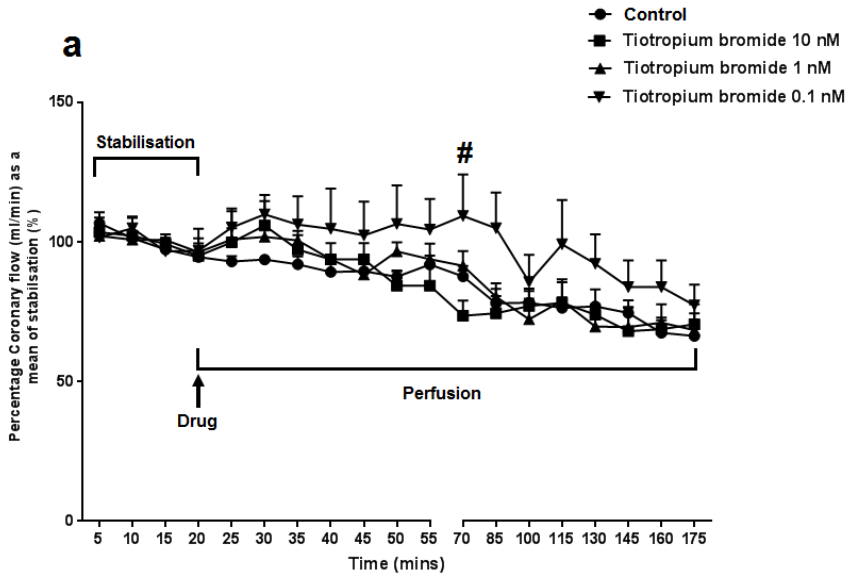
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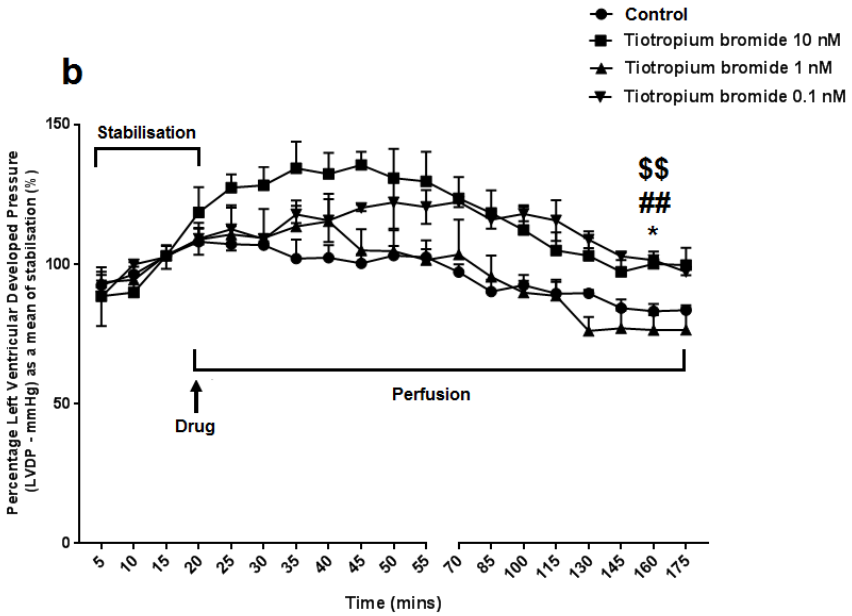
Control
Tiotropium bromide 10 nM
Tiotropium bromide 1 nM
Tiotropium bromide 0.1 nM
Stabilisation
#
Perfusion
Drug
Percentage Coronary flow (ml/min) as a mean of stabilisation (%)
Time (mins)


b
Control
Tiotropium bromide 10 nM
Tiotropium bromide 1 nM
Tiotropium bromide 0.1 nM
Stabilisation
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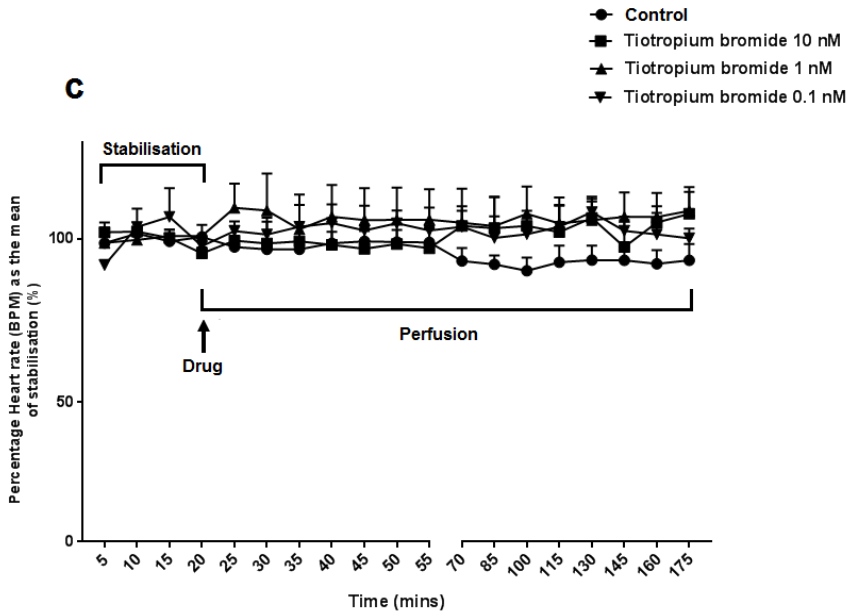
Perfusion
Drug
Percentage Left Ventricular Developed Pressure (LVDP - mmHg) as a mean of stabilisation (%)
Time (mins)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Control
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Tiotropium bromide 1 nM
Tiotropium bromide 0.1 nM
Stabilisation
Perfusion
Drug
Percentage Heart rate (BPM) as the mean of stabilisation (%)
Time (mins)

Figure 1. The effect of Tiotropium bromide (10 – 0.1 nM) on the haemodynamic function of the heart. **A)** Tiotropium bromide effect on coronary flow, **B)** LVDP and **C)** Heart rate as a percentage of the mean stabilisation period. * p<0.05 vs. time matched control, # p<0.05 Tiotropium bromide (0.1 nM) vs. Tiotropium bromide (10 nM), ## p<0.01 Tiotropium bromide (1 nM) vs. Tiotropium bromide (10 nM), $$ Tiotropium bromide (1 nM) vs. Tiotropium bromide (0.1 nM) n = 3-5. BPM, beats per minute.

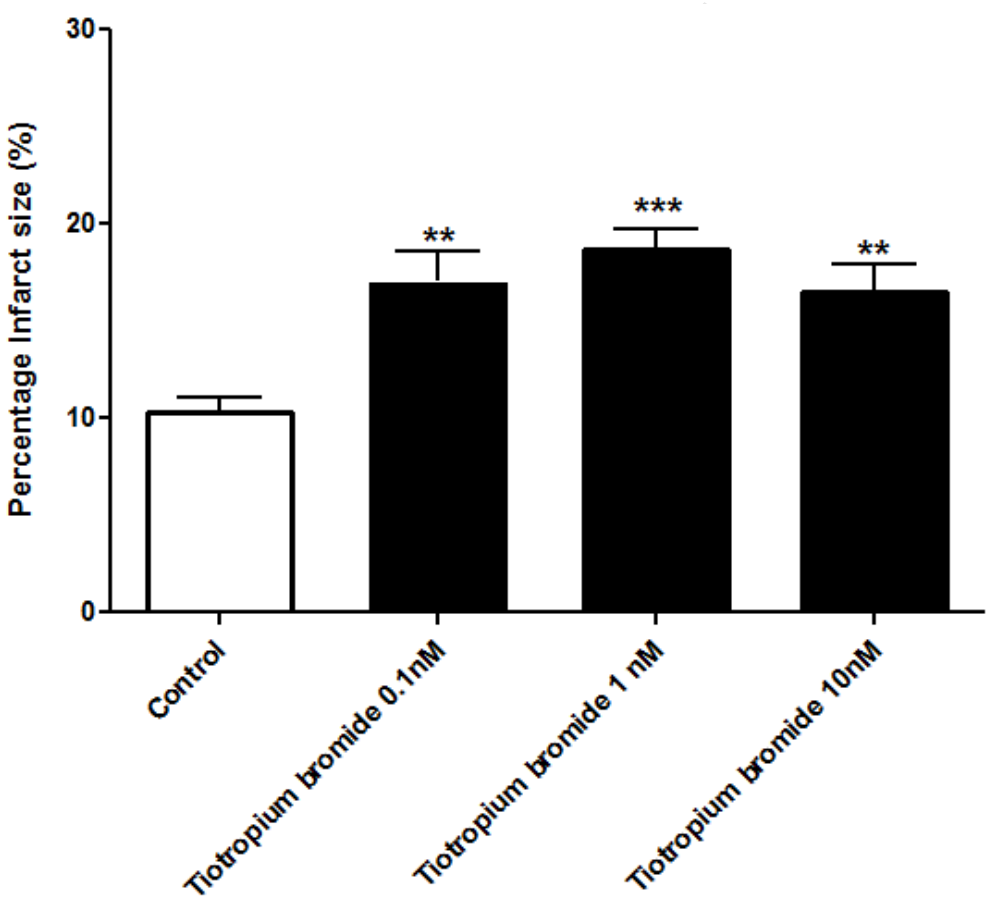
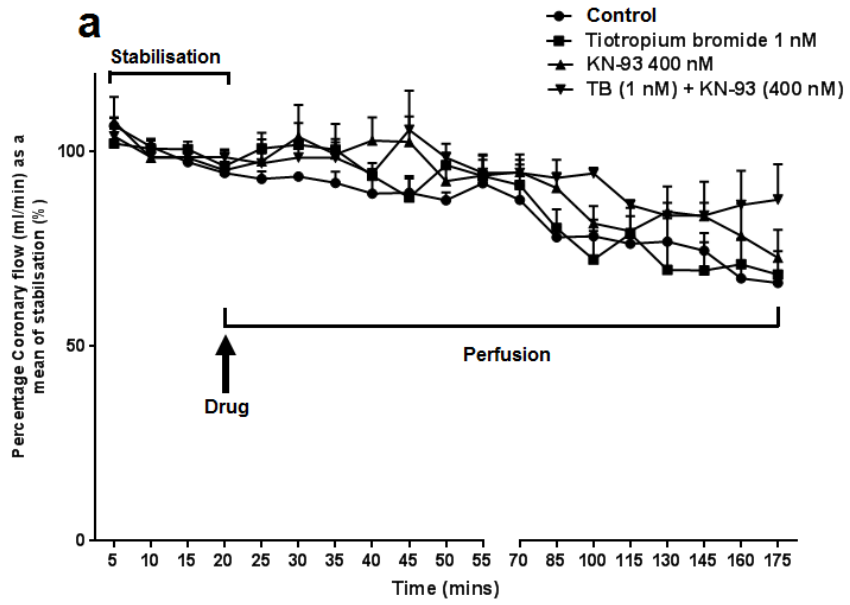
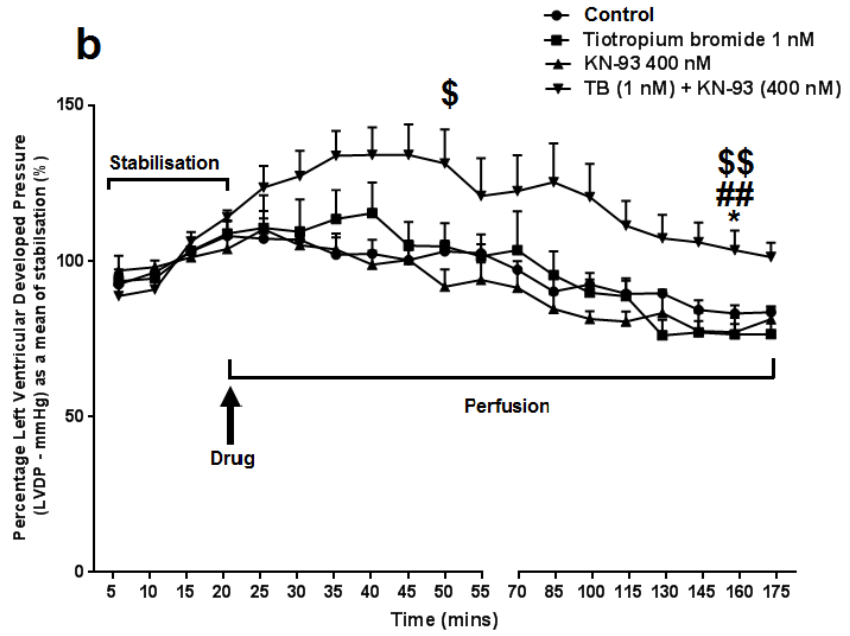


Figure 2. The effect of Tiotropium bromide (10 – 0.1 nM) on infarct size in the whole heart Langendorff model. ** p<0.01 vs. Control, and *** p<0.001 vs. Control, n = 3-5.


a
Control
Tiotropium bromide 1 nM
KN-93 400 nM
TB (1 nM) + KN-93 (400 nM)
Stabilisation
Perfusion
Drug
Percentage Coronary flow (ml/min) as a mean of stabilisation (%)
Time (mins)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Control
Tiotropium bromide 1 nM
KN-93 400 nM
TB (1 nM) + KN-93 (400 nM)
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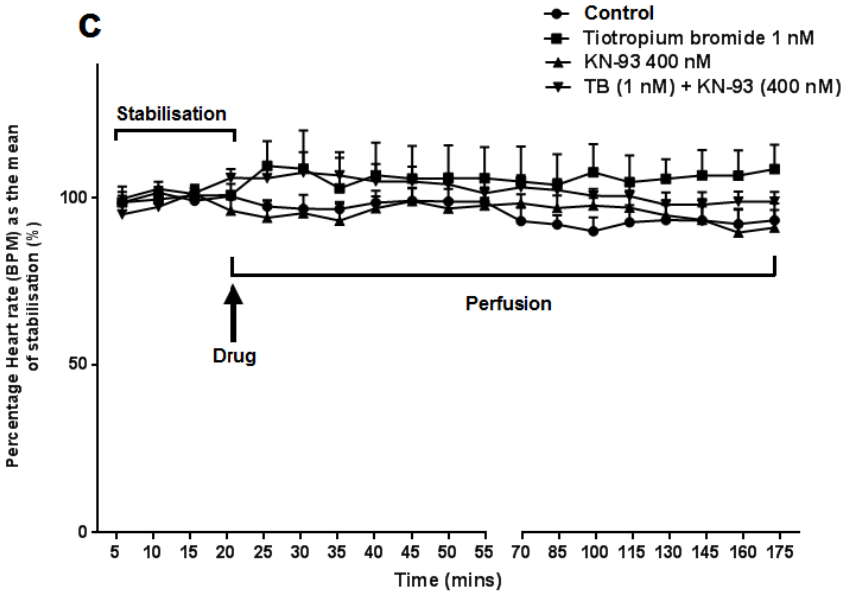
Stabilisation
Perfusion
Drug
Percentage Left Ventricular Developed Pressure (LVDP - mmHg) as a mean of stabilisation (%)
Time (mins)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Control
Tiotropium bromide 1 nM
KN-93 400 nM
TB (1 nM) + KN-93 (400 nM)
Stabilisation
Perfusion
Drug
Percentage Heart rate (BPM) as the mean of stabilisation (%)
Time (mins)

Figure 3. The effect of Tiotropium bromide (1 nM) ± KN-93 (400 nM) on the haemodynamic function of the heart. **A)** Tiotropium bromide ± KN-93 effect on coronary flow, **B)** LVDP and **C)** Heart rate as a percentage of the mean stabilisation period. * p<0.05 vs. time matched control, ## p<0.01 TB (1 nM) + KN-93 (400 nM) vs. Tiotropium bromide (1 nM), $ p<0.05 TB (1 nM) + KN-93 (400 nM) vs. KN-93 (400 nM) and $$ p<0.01 TB (1 nM) + KN-93 (400 nM) vs KN-93 (400 nM) n = 4-5. BPM, beats per minute, TB, Tiotropium bromide.

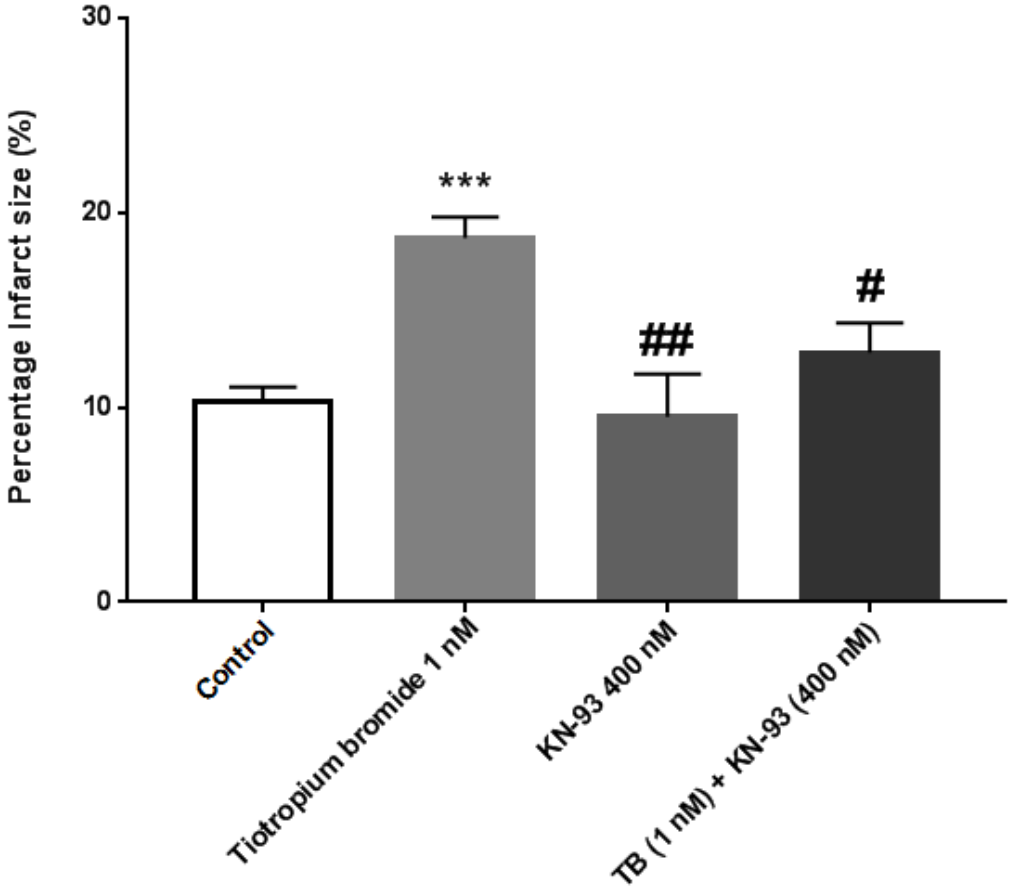

Figure 4. The effect of Tiotropium bromide (1 nM) ± KN-93 (400 nM) on infarct size in the whole heart Langendorff model. *** p<0.001 vs. Control, # p<0.05 vs. Tiotropium bromide (1 nM), ## p<0.01 vs. Tiotropium bromide (1 nM), n = 4-5. TB, Tiotropium bromide.

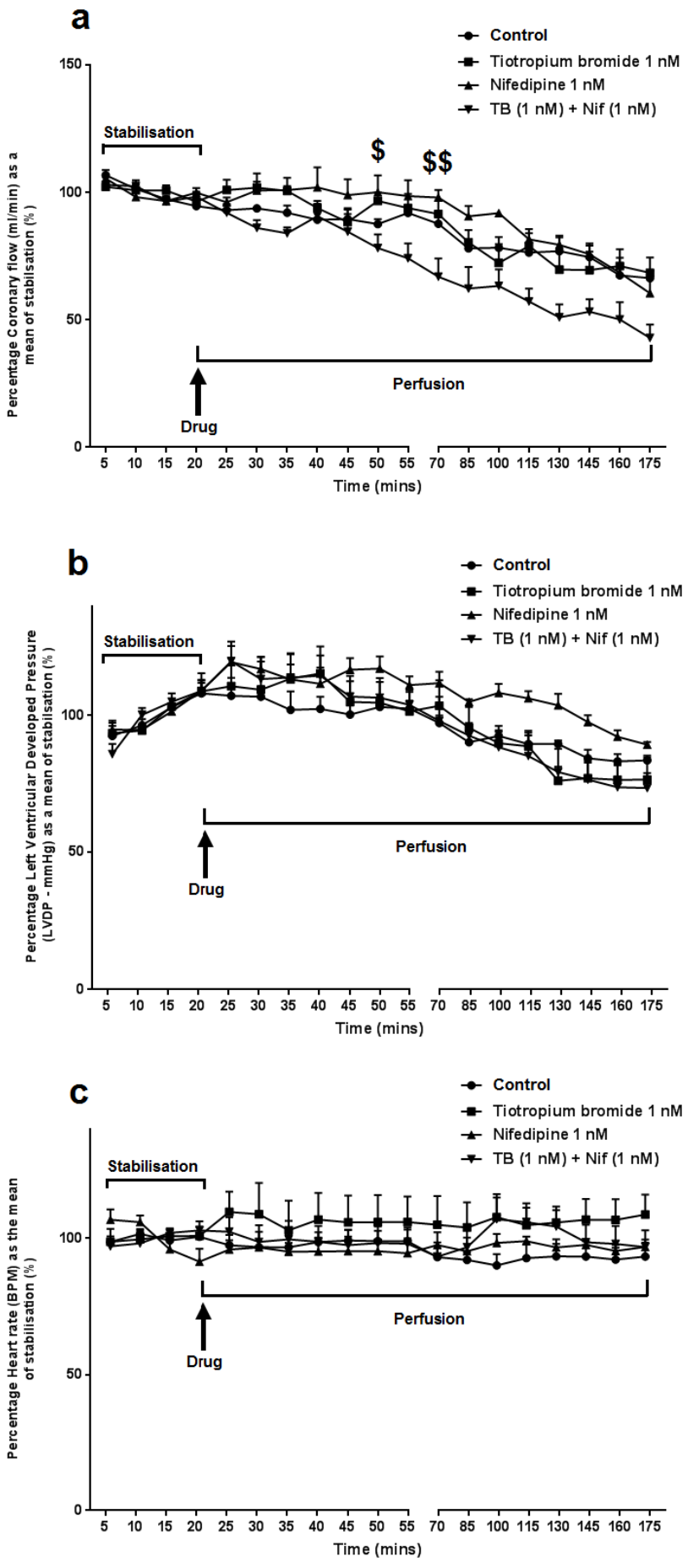
a
Control
Tiotropium bromide 1 nM
Nifedipine 1 nM
TB (1 nM) + Nif (1 nM)
Stabilisation
$
$$
Perfusion
Drug
Percentage Coronary flow (ml/min) as a mean of stabilisation (%)
Time (mins)
b
Control
Tiotropium bromide 1 nM
Nifedipine 1 nM
TB (1 nM) + Nif (1 nM)
Stabilisation
Perfusion
Drug
Percentage Left Ventricular Developed Pressure (LVDP - mmHg) as a mean of stabilisation (%)
Time (mins)
c
Control
Tiotropium bromide 1 nM
Nifedipine 1 nM
TB (1 nM) + Nif (1 nM)
Stabilisation
Perfusion
Drug
Percentage Heart rate (BPM) as the mean of stabilisation (%)
Time (mins)

Figure 5. The effect of Tiotropium bromide (1 nM) ± Nifedipine (1 nM) on the haemodynamic function of the heart. **A)** Tiotropium bromide ± Nifedipine effect on coronary flow, **B)** LVDP and **C)** Heart rate as a percentage of the mean stabilisation period. $ p<0.05 TB (1 nM) + Nif (1 nM) vs. Nif (1 nM) and $$ p<0.01 TB (1 nM) + Nif (1 nM) vs. Nif (1 nM) n = 4-5. BPM, beats per minute, TB, Tiotropium bromide, Nif, Nifedipine.

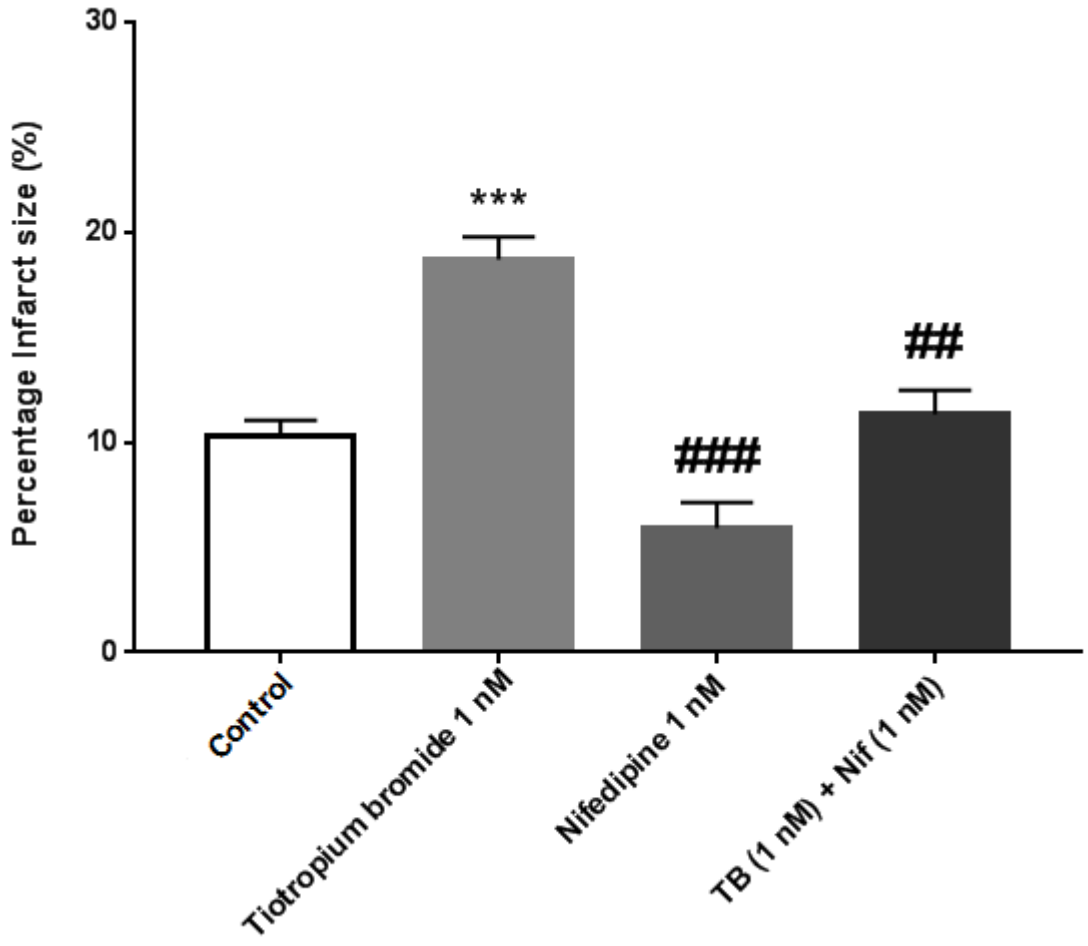

Figure 6. The effect of Tiotropium bromide (1 nM) ± Nifedipine (1 nM) on infarct size in the whole heart Langendorff model. *** p<0.001 vs. Control, ## p<0.01 vs. Tiotropium bromide (1 nM), ### p<0.001 vs. Tiotropium bromide (1 nM), n = 4-5. TB, Tiotropium bromide, Nif, Nifedipine.

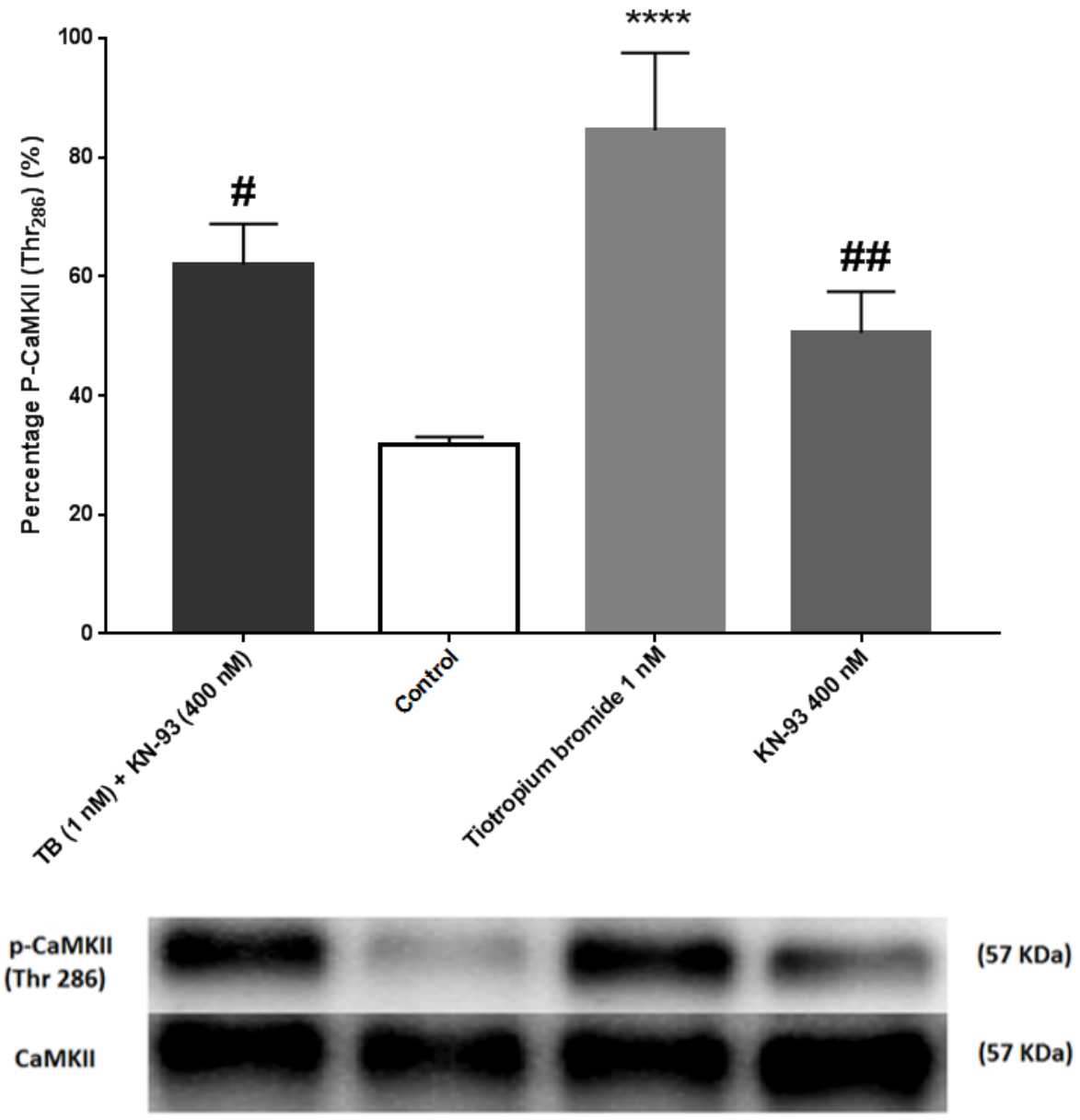

Figure 7. The effect of Tiotropium bromide (1 nM) ± KN-93 (400 nM) on expression of phosphorylated cardiac CaMKII. *** p<0.001 vs. Control, # p<0.05 vs. Tiotropium bromide (1 nM), ## p<0.01 vs. Tiotropium bromide (1 nM), n = 3. TB, Tiotropium bromide.

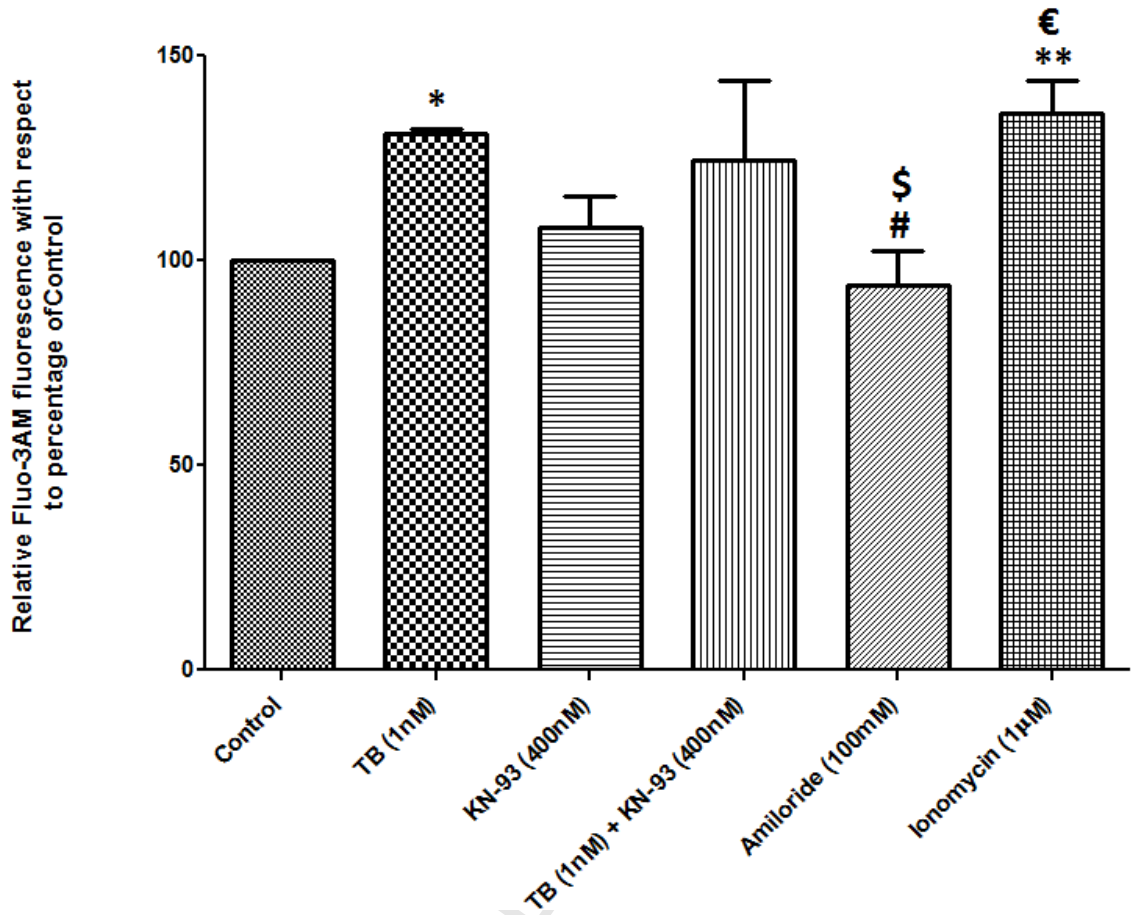

Figure 8. Calcium release as determined with Fluo 3-AM fluorescence following Tiotropium bromide (1 nM) ± KN-93 (400 nM) in cardiomyocytes. * p<0.05 vs. Control, ** p<0.01 vs. Control, # p<0.05 vs. TB (1 nM), $ p<0.05 vs. TB (1 nM) + KN-93 (400 nM), € vs. p<0.05 vs. Amiloride (100 mM), n = 3-6. TB, Tiotropium bromide.

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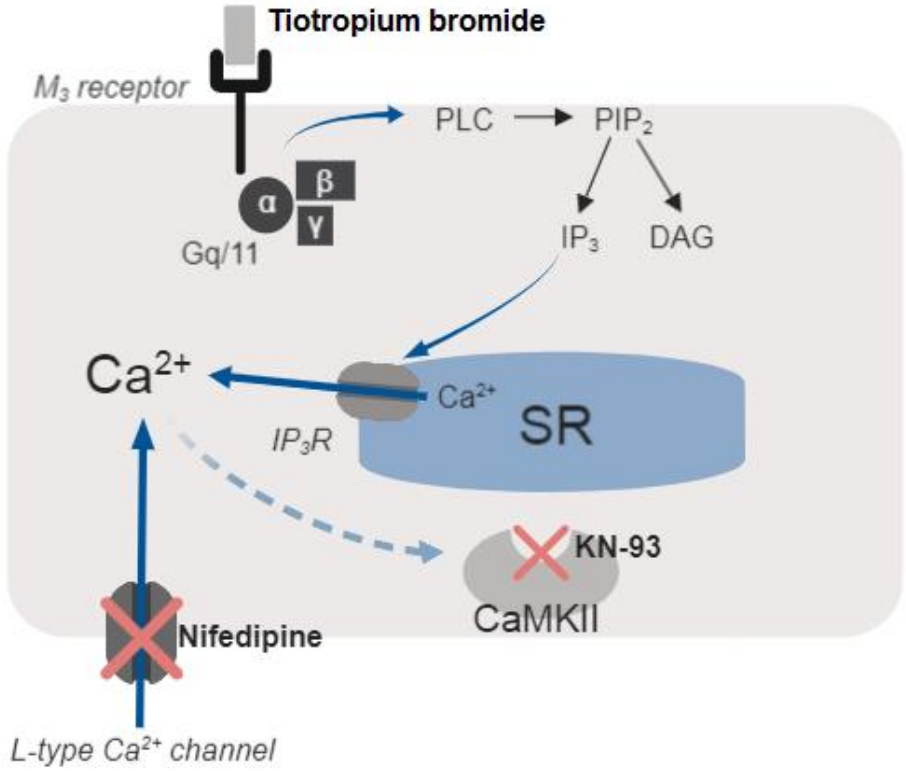

Figure 9. A schematic illustrating the proposed effect of Tiotropium bromide administration in cardiomyocytes. Tiotropium bromide may exert an inverse agonist function and trigger calcium signalling through Gq/11 mediated phospholipase C (PLC) signalling releasing sarcoplasmic reticulum (SR) Ca2+ into the cell. PIP2, phosphatidylinositol 4,5 bisphosphate; IP3, inositol 1,4,5 triphosphate; DAG, diacylglycerol; M3, muscarinic receptor subtype 3; IP3R, inositol 1,4,5 triphosphate receptor. Created using BioRender software.

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Highlights:

- Anti-muscarinics have been associated with increased cardiovascular risk in chronic obstructive pulmonary disease (COPD).
- Tiotropium bromide results in detrimental calcium signalling in whole heart models, observed with increased infarction.
- The effects of Tiotropium bromide are reversed with L-type Ca^{2+} channel and CaMKII inhibitors.
- Anti-muscarinics such as Tiotropium bromide may result in adverse cardiac effects without affecting cardiac function.
- Further tests should be used to monitor COPD patients using anti-muscarinics for adverse cardiovascular effects.

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