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Research Article

Intracellular stored calcium plays a minor role in G_q-coupled receptor-mediated contraction in rat airway smooth muscle

Kayatri Govindaraju[1], Mei Kee Lee[1], Yvonne Mbaki[2] and Ting Kang Nee[1]*

[1] *Department of Biomedical Sciences, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia.*
[2] *School of Life Sciences, University of Nottingham, Medical School Queen's Medical Centre, Nottingham NG72UH, United Kingdom.*

Abstract

The general notion of activation of G_q-protein coupled receptors (GPCR) involves the mobilisation of stored and extracellular calcium and leads to smooth muscle tissue contraction. The aim of this study was to investigate the involvement of calcium mediated contractions in vascular and airway smooth muscles. Using standard organ bath procedures, aortic and tracheal rings were obtained from 6 to 8 week-old male Sprague Dawley rats. To activate the G_q protein receptors, phenylephrine (PE), an α_1-adrenoceptor agonist, and carbachol, a M_3 cholinoceptor agonist was added to baths containing the aortic and tracheal rings, respectively. The maximum response (E_{max}) to PE was reduced from 158.8 $\pm$ 11.8% (n=6) to 62.5 $\pm$ 12.4 % (n=8) upon removal of extracellular calcium in Krebs-Ringer solution. Maximal response to PE was also suppressed in the presence of nifedipine, a L-type Ca^{2+} channel inhibitor, (70.3 $\pm$ 11 %, n=8) and SKF96365, a canonical transient receptor potential cation channel inhibitor, (26.7 $\pm$ 13.2 %, n=5) when the influx of extracellular calcium was blocked. Removal of stored calcium also attenuated the PE contraction (p<0.05). Contractile responses to carbachol in the airway were totally abolished in the absence of calcium in the Krebs-Ringer solution (208.6 $\pm$ 23 % [n=8] vs 10.7 $\pm$ 4.2 % [n=3]). This was different from the aorta where a measurable response was detected despite the absence of external calcium. Blockage of extracellular calcium influx in the presence of nifedipine and SKF96365 also showed similar lack of responses in the trachea. Interestingly, removal of stored calcium did not affect the carbachol responses (p>0.05). From these observations, we conclude that the role of stored and extracellular calcium in G_q protein activation is not the same across different types of smooth muscle tissues.

Keywords: Phenylephrine, carbachol, stored and extracellular calcium, aorta, trachea

1.0   Introduction

Smooth muscle contraction is regulated by the changes in the concentration of free Ca^{2+} in the cytosol. An increase in free intracellular Ca^{2+} results from either 1) increased flux of Ca^{2+} into the cell e.g. through Ca^{2+} channels in the plasma membrane under the control of membrane depolarisation or upon agonist stimulation or 2) by release of Ca^{2+} from internal stores e.g. sarcoplasmic reticulum (SR), which is controlled by second messengers. The free Ca^{2+} in the cytoplasm binds to a calcium binding protein called calmodulin to form a calcium-calmodulin complex. The calcium-calmodulin complex activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chains (MLC). MLC phosphorylation leads to cross-bridge formation between the myosin and actin filaments, and this is followed by smooth muscle contraction. In contrast, as the Ca^{2+} concentration begins to decline, Ca^{2+}-sensitization of the contractile proteins is signalled by the RhoA/Rho-kinase pathway to inhibit the dephosphorylation of MLC phosphatase (MLCP) to maintain the force generation. Removal of Ca^{2+} from the cytosol and stimulation of MLCP initiates the process of smooth muscle relaxation (Wynne *et al*., 2009). The degree of MLC phosphorylation and the vascular smooth muscle contractile tone is regulated by G-protein-coupled signal transduction pathways and by nitric oxide activation of guanylyl cyclase and cyclic guanine monophosphate (cGMP) formation.

GPCRs respond to various extracellular signals and transduce them to heterotrimeric G proteins, and play an important role in various signalling pathways. Heterotrimeric G proteins (Gα, Gβ/Gγ subunits) constitute one of the most important components of the cell signalling cascade (Tuteja, 2009). Gα proteins are divided into four groups according to their structural and functional similarities; G_s, G_i, G_q, and G_{12}. The G_s and G_i families regulate adenylyl cyclase activity, while G_q activates phospholipase Cβ and $G_{12/13}$ and can activate small guanine tri-phosphatases (GTP) families. The G_q family consists of four members: G_q, G_{11}, G_{14}, and $G_{15/16}$ and their respective α subunits and are thus Gα_q, Gα_{11}, Gα_{14}, and G$\alpha_{15/16}$ (Billington and Penn, 2003). Most of the specificity of signalling resides in the Gα subunit.

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* Correspondence: Department of Biomedical Sciences, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia.
e-Mail: Kang-Nee.Ting@nottingham.edu.my

In the most common signalling pathway, activation of a receptor coupled to G_q protein results in the activation of phospholipase C (PLC), which leads to the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG), hence, releasing Ca^{2+} from intracellular and extracellular milieu, respectively. The second-messengers (IP_3 and DAG) play an important role in smooth muscle contraction by mobilising Ca^{2+} from multiple signalling pathways involved in agonist-induced contraction (Abdel-Latif, 2001). G_q signalling can also activate Rho/Rho-kinase (Rho/ROCK) signalling pathways via Rho-guanine nucleotide exchange factors (Rho-GEF). In order to explore the physiological role of Ca^{2+} in smooth muscles and how it is regulated in different tissues, we selected rat aorta and trachea which express α_1-adrenoceptor and M_3 muscarinic receptors respectively, and are coupled to G_q-protein.

Some earlier studies have suggested that noradrenaline stimulating the α_1-adrenoceptor of the rat tail artery maintains its contraction by asynchronous repetitive SR Ca^{2+} release rather than by sustained Ca^{2+} influx (Iino *et al.,* 1994) . However, Lee *et al.*, (2001) challenged this view by reporting that, the initial phase of phenylephrine (PE) contraction in rabbit interior vena is initiated by Ca^{2+} release from the SR, and the tonic phase is supported by sustained Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels (L-type VGCCs) and/or receptor-operated channels (ROCs). The M_3-muscarinic receptor on the other hand, activates both intracellular and extracellular Ca^{2+} upon stimulation to cause contraction in human urinary smooth muscle bladder (Visser and van Mastrigt, 2000). However, in the guinea-pig urinary smooth muscle, carbachol-induced contraction mediated by M_3-muscarinic receptors was predominantly dependent on extracellular Ca^{2+} (Rivera and Brading, 2006) which opposes the previous findings in humans. The contribution of intracellular and extracellular Ca^{2+} on PE- and carbachol-induced contraction in rat aorta and trachea has not been fully described. To date, it is however known that the contraction of smooth muscle to PE and carbachol involves both intracellular and extracellular Ca^{2+}. We therefore aimed to investigate the role of extracellular and stored Ca^{2+} on G_q coupled receptor activation in vascular and airway smooth muscles.

2.0   Materials and methods

2.1   *Drugs and Krebs-Ringer bicarbonate solution*

(R)-(−)-phenylephrine hydrochloride (Sigma), carbachol (Nacalai Tesque, Japan), 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole (SKF 96365) (Sigma, USA), nifedipine (Nacalai Tesque, Japan), thapsigargin (Sigma, USA) and potassium chloride (KCl) were used. All drugs were dissolved in distilled water to prepare 0.1M stock concentration except SKF 96365, nifedipine and thapsigargin which were dissolved in dimethyl sulfoxide (DMSO) before subsequent dilutions in distilled water (final bath concentration of the solvent was <0.2 % (v/v)). The Krebs-Ringer solution was freshly prepared daily, following the composition (in mM): NaCl 120, KCl 5.4, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.26 and gassed with 95 % O_2, 5% CO_2. Calcium free Krebs-Ringer solution was made in the similar composition as normal Krebs-Ringer solution but without the inclusion of $CaCl_2$ in the solution. The chelator ethylene glycol tetraacetic acid (EGTA), 0.1 mM was added to calcium free Krebs-Ringer solution, as a perfusion buffer in experiments to evaluate the role of external calcium.

2.2   *Tissue preparation*

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB REF: UNMC2kn). All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Male Sprague Dawley rats (235-440 g; 2-3 months old) purchased from University Putra Malaysia, were sacrificed on the day of experiment. Rat aorta and trachea were removed and excised into 4 mm and 2 mm rings, respectively. The methods used in this study were adopted from (Loong *et al.*, 2015). Changes in muscle tension responses produced by tissue contraction were detected by a force transducer (MLTF050/ST, ADInstruments, US). Data were recorded by a PowerLab data acquisition system (LabChart v7.3.4) and a computer (Hewlett-Packard, US). Muscle tension was expressed in milliNewtons (mN). Tissues were left to equilibrate to bath conditions for 30 minutes after the application of 9.8 mN tension to achieve a steady baseline tone. Following equilibration, all tissues were exposed twice to 60 mM potassium chloride (KCl) to assess tissue viability and to provide reference contractions for subsequent data analysis. After the KCl was washed out and a stable basal tone was re-established, experiments were carried out as in section 2.3.

2.3   *Organ bath assembly*

Investigation of the role of external calcium on G_q coupled receptor activation

Aortic and tracheal segments were tested with PE and carbachol, respectively. Concentration-response curves (CRC) to the agonists were constructed in both normal Krebs-Ringer solution and calcium free Krebs-Ringer solution. The degree of response (tissue contraction) was measured as tension weight of the contraction achieved by the increasing agonist concentrations.

In a separate protocol, to further deduce the effect of extracellular calcium on PE and carbachol-induced contraction, the aortic rings were pre-incubated with nifedipine (10 μM) or SKF 96365 (300 μM), while the tracheal rings were pre-incubated with nifedipine (100 μM) or SKF 96365 (100 μM) for at least 40 minutes in normal Krebs-Ringer solution, prior to the construction of the agonist concentration-response curve (CRC). The concentration of each inhibiting agent (nifedipine and SKF 96365) was chosen on the basis of its ability to cause between 70% and 90% reduction in PE/CCH-induced tone. This was determined in our earlier experiments (data not shown). Tissue contraction to carbachol or PE was expressed as a percentage of 60 mM KCl-induced tone.

Examination of the role of stored calcium following G_q coupled receptor activation

Following the second KCl stimulation, tissues were washed with calcium free Krebs-Ringer solution containing 0.1mM of EGTA. The tissues were then incubated with thapsigargin (1 μM) for at least 40 minutes. To empty residual cytosolic calcium, phenylephrine (10 μM) was thereafter added. The tissues were then washed with normal Krebs-Ringer solution followed by incubation of thapsigargin (1 μM) for the second time. This ensured the intracellular stores were emptied completely. After incubating for at least 40 minutes, CRCs of PE were constructed in rat isolated aorta. The same protocol was conducted on rat isolated trachea with thapsigargin (1 μM) and carbachol (1 μM) to study the role of intracellular calcium in airway smooth muscle. Tissue contraction to PE or carbachol was expressed as a percentage of 60 mM KCl-induced tone.

2.4   **Statistical Analysis**

Data were analysed and graphs were drawn using PRISM v7.0 (GraphPad software). All data were expressed as mean ± standard error of mean (SEM) of n number of animals. Maximum tissue contraction or relaxation response (E_{max}) and concentration to produce basal response by 50% (EC_{50}) was derived from nonlinear regression analysis of the obtained CRC. Statistical analyses were performed using Student's unpaired t-test. Results were considered statistically significant if $P < 0.05$.

3.0   **Results**

3.1   *Activation of a G_q coupled receptor α_1-adrenoceptor) in vascular smooth muscle involve mobilization of stored and extracellular calcium*

In order to examine the role of extracellular calcium on activation of α-adrenoceptor contraction, PE CRC was performed in normal or calcium free Krebs-Ringer solutions. The E_{max} of PE in normal Krebs-Ringer solution was 158.8 ± 11.8 % (n=6) and in calcium-free Krebs Ringer solution was 62.5 ± 12.3 % (n=8) (see Fig.1A). To further confirm the role of extracellular Ca^{2+} in the PE-induced contractile response, nifedipine (a L-type calcium channel blocker) was added to the bath prior to the construction of the PE CRC. The E_{max} of the PE CRC was reduced to 70.3 ± 11 % (n=8). The presence of the canonical transient receptor potential cation (TRPC) channel blocker, SKF 96356, attenuated the PE E_{max} to 26.7 ± 13.2 % (n=5) (see Fig.2A). These results inferred that the maximum contractile response to a G_q coupled receptor was significantly suppressed in the absence of external calcium influx into cells. Subsequent investigations of the role of stored calcium using thapsigargin (SERCA pump inhibitor) to deplete the sarcoplasmic calcium store demonstrated a significant reduction in the PE-induced contractile response ($E_{max} = 65.1 \pm 8.9$ % (n=5) (unpaired Student's *t*-test, two-tailed *p=0.0002)* (see Fig.3A and 3C).

3.2   *Activation of a G_q coupled receptor (M_3 cholinoceptor) in airway smooth muscle involved modulation of extracellular calcium.*

In calcium free Krebs Ringer solution, the contractile response to carbachol was markedly reduced when compared to normal Krebs-Ringer solution with measured E_{max} of 10.7 ± 4.2 %, (n=3) and 208.6 ± 23 % (n=8) respectively (see Fig.1B). Both nifedipine and SKF 96365 significantly attenuated the carbachol E_{max} to 83.7 ± 14 % (n=5) and 16.7 ± 6.1 % (n=5), respectively (see Fig. 2B). Thapsigargin treatment did not seem to affect the maximal response of carbachol-induced contraction as compared to normal Krebs-Ringer solution with the attainment of an E_{max} value of 167.8 ± 8.8 %, (n=4) (unpaired Student's *t*-test, two-tailed *p=0.25)*, but caused a significant leftward shift of the carbachol-CRC with EC_{50} value of $(0.16 \pm 0.009$ μM, n=4) (unpaired Student's *t*-test, two-tailed *p=0.02)* (see Fig.3B and 3D).
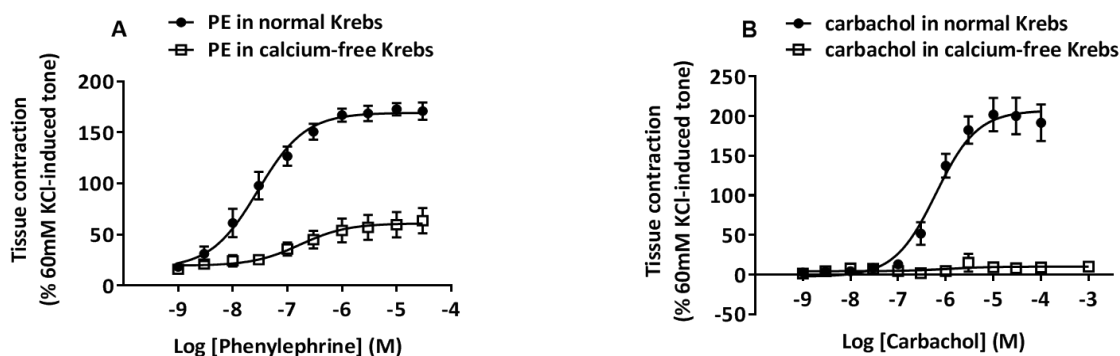

Figure 1: Effect of normal and calcium free Krebs solution on phenylephrine- or carbachol- concentration response curve on rat isolated (A) aorta and (B) trachea. Tissue responses have heen expressed as a percentage of 60mM KCl-induced contraction and are shown as means ± SEM of 3 to 8 animals.
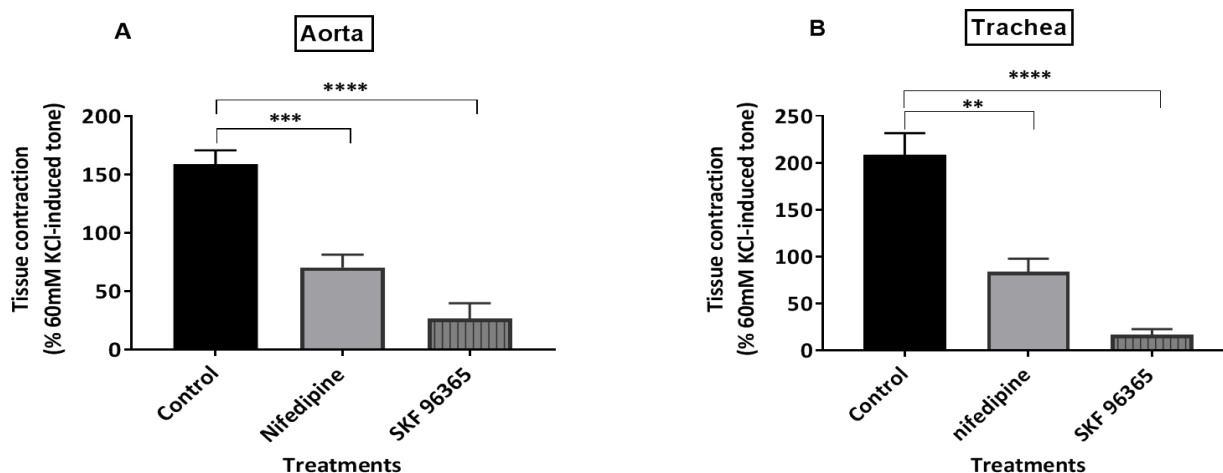

Figure 2: Effect of nifedipine and SKF 96365 pre-incubation on maximal response (E_{max}) of (A) phenylephrine concentration response curve in rat aortic rings and (B) carbachol-concentration response curve in tracheal rings. Tissue responses have been expressed as a percentage of 60mM KCl-induced contraction and are shown as means ± SEM of 3 to 8 animals (unpaired t-test :**p<0.01, ***p<0.001, ****p<0.0001).
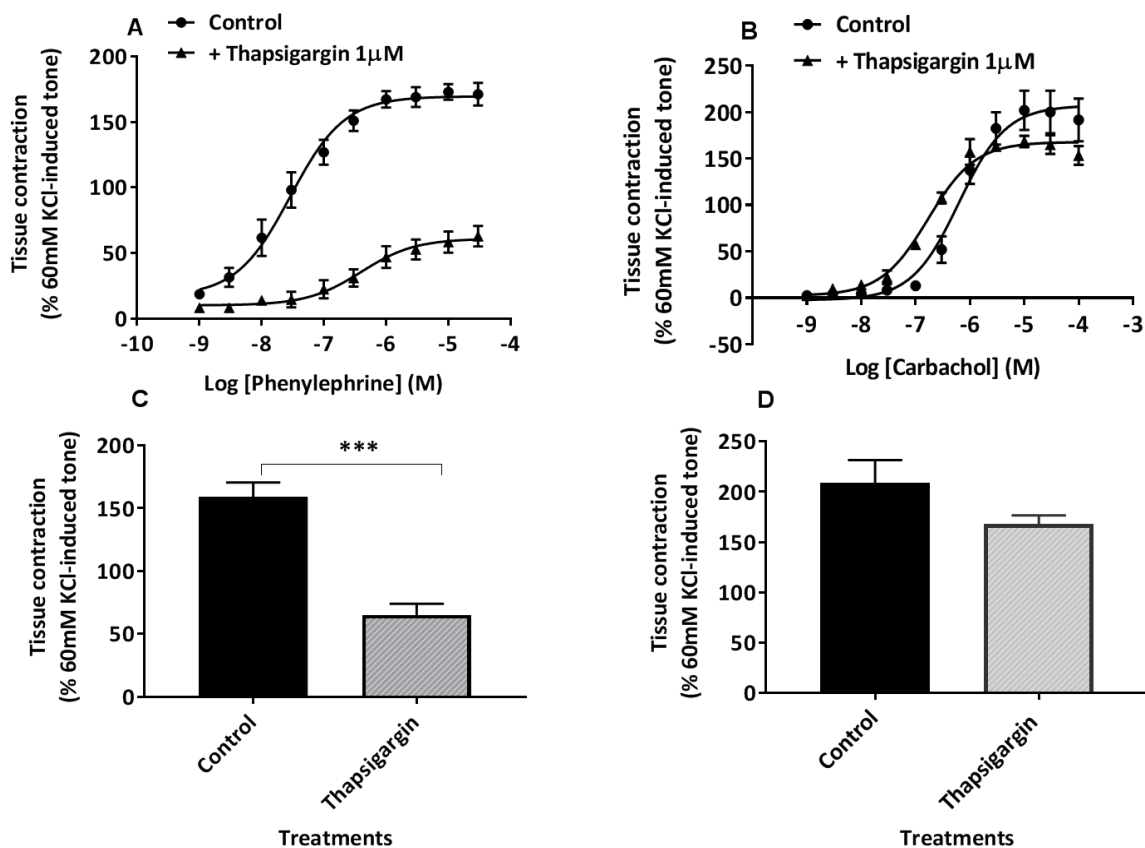
Figure 3: Effect of thapsigargin pre-incubation on agonist concentration response curves (CRCs) in rat isolated (A) aorta and (B) trachea. Maximum response (E_{max}) of agonist CRCs between control and in the presence of thapsigargin are depicted in (C) aorta and (D) trachea. Tissue responses have been expressed as a percentage of 60mM KCl-induced-contraction and are shown as means ± SEM of 3 to 8 animals (unpaired t-test : ***$p < 0.001$).

4.0   Discussion

The present study examined the role of extracellular and stored Ca^{2+} upon activation of G_q-coupled receptors in aorta and airway smooth muscles. Although G_q protein was activated in both tissues, the utilisation of stored Ca^{2+} is not the same.

Stimulation of G_q-coupled receptors leads to the release of stored and influx of extracellular Ca^{2+} via second messengers IP_3 and DAG respectively. DAG via PKC activation, opens the canonical transient receptor potential cation (TRPC) channels to allow Ca^{2+} influx through TRPC3, TRPC6 or TRPC7 (Hoffman et al., 1999). The initial influx of Ca^{2+} via the TRPC channels, depolarises the membrane and leads to opening of L-type VGCC. In addition to this paradigm, the downward signalling also involves activation of RhoA, a mediator of calcium sensitisation. Active RhoA interacts with Rho GEF to stimulate the downstream effector, ROCK and PKC-related kinase which contributes to smooth muscle contraction by involving the release of intracellular Ca^{2+} (Szasz and Webb, 2017).

All G-proteins have a similar structure and they operate in a similar way (Alberts et al., 2002). However, it has been shown that many of the cellular responses mediated by GPCRs do not only involve stimulation of conventional second-messenger-generating systems, instead result from the functional integration of an intricate network of intracellular signalling pathways (Marinissen and Gutkind, 2001).

In the case of the aorta, we found a marked reduction of PE contractility in calcium free medium as compared to PE in normal Krebs-Ringer solution. Despite the large reduction in the E_{max} responses in the calcium free medium, the contraction to PE was still present and measureable. This suggests that extracellular Ca^{2+} is important in PE-induced contraction but it is not the only source of Ca^{2+} required to elicit contraction in the vascular tissue. On the contrary, stimulation of G_q protein by carbachol via M_3 cholinoceptor did not elicit any contraction in the absence of external calcium in tracheal smooth muscle. In rat trachea, M_2 and M_3 cholinoceptor subtypes are co-expressed and contraction is mediated primarily by the M_3-cholinoeceptor which is coupled to G_q protein (Preuss and Goldie, 1999). This interesting observation shows that without external Ca^{2+} in the medium, activation of G_q protein receptor alone cannot elicit any measurable contraction in the airway smooth muscle (ASM).

Activation of the G_q protein in the smooth muscle involves a downstream Ca^{2+} influx through the TRPC and L-type VGCC (Iino et al., 1994; Lee et al., 2001; Hoffmann et al., 1999). Blocking of the TRPC channels by SKF 96365 markedly reduced contractions in both tissues. Nifedipine which blocks the L-type VGCC also reduced contractions in aorta and trachea but at a lower magnitude when compared to blockage of TRPC channels. This shows that the TRPC channels play a crucial role in the initial influx of Ca^{2+} prior to the second phase of Ca^{2+} entry via the L-type VGCC. This initial influx of Ca^{2+} promotes membrane depolarisation which opens up the L-type VGCC. It is well established that L-type VGCC is essential for coupling membrane depolarization to the influx of Ca^{2+} in all excitable cells (Alexander et al., 2017). Blockage of this channel by nifedipine stops external Ca^{2+} influx leading to the loss of sustained contractions as shown in our experiments. Our findings confirm the role of TRPC channels and L-type VGCC are crucially important in eliciting contraction mediated by G_q protein activation in both, rat aortic and tracheal smooth muscles.

The role of stored Ca^{2+} in regulating contractions in both types of tissues was investigated by using thapsigargin to empty the internal calcium store. Removal of stored Ca^{2+} affected the maximum response to PE in the aorta but it had no effect on carbachol induced contraction in the trachea. This finding supports our earlier observation that activation of G_q protein in the vascular tissue requires mobilisation of both external and stored Ca^{2+}. Stored Ca^{2+} plays an important role in the vasculature to maintain a certain tone for tight regulation of blood pressure

(Leung *et al.*, 2008). However, stored Ca^{2+} does not seem to have such an important physiological role in the airways. ASM in general maintains a relatively low intracellular calcium concentration under resting conditions (Jude *et al.*, 2008), thus, providing a reasonable explanation for the major contribution of extracellular Ca^{2+} in carbachol-induced contraction in rat trachea. This observation is consistent with a study previously done on guinea-pig trachea (Takemoto *et al.*, 1998), who also reported that carbachol-induced contraction was not affected by thapsigargin.

Vascular smooth muscle cells (VSMC) are a type of specialized smooth muscle cells, which reside within the medial layer of blood vessels. The primary function of VSMC is to control blood vessel diameter in response to intraluminal pressure and various physiological stimuli emanating from the central nervous system, kidneys, and local tissues (Lin *et al.*, 2016). Previously, Lee *et al.*, (2001) concluded that the refilling of the SR Ca^{2+} store through SERCA pump, which is maintained by extracellular Ca^{2+} entry is essential in sustaining PE-induced contraction. However, our present study shows that the intracellular Ca^{2+} alone in the absence of extracellular Ca^{2+} entry, may be sufficient to cause contraction and sustain PE-induced contraction in the rat aorta, further reiterating the importance of stored Ca^{2+} in maintaining the vascular tone. This is in accordance with recent studies which have shown that IP_3R-mediated Ca^{2+} release in VSMC is a critical player in cellular contraction (Lin *et al.*, 2016).

5.0 Conclusion

Conventionally, activation of a G_q protein will elicit entry of extracellular Ca^{2+} and release of stored Ca^{2+} to produce contraction in smooth muscle cells. This cellular mechanism is not known to be tissue specific. However, our study suggests that the participation of these two sources of Ca^{2+} in muscle contraction is not the same in different types of smooth muscles. It is shown that the role of external Ca^{2+} and stored Ca^{2+} in the rat trachea is not the same as in the aorta. External and stored Ca^{2+} synergistically promote contraction in the vascular tissue like aorta but removal of either source still enables a tone of the tissue to be maintained. On the other hand, ASM does not respond to G_q receptor activation in the absence of extracellular Ca^{2+} whilst removal of stored Ca^{2+} has no effect on the contractile responses. From these observations, we conclude that the role of stored and extracellular calcium in G_q protein activation is not the same across different type of smooth muscle tissues.

6.0 Declaration

The authors declare no conflicts of interest in this work.

7.0 Acknowledgements

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