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Prominent role of intracellular Ca^{2+} release in hypoxic vasoconstriction of canine pulmonary artery

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1 The possible role of sarcoplasmic reticulum (SR) Ca^{2+} stores in hypoxic pulmonary vasoconstriction (HPV) is not well understood. In order to assess the possible role of intracellular Ca^{2+} release from SR Ca^{2+} stores in HPV, we examined the effects of: (1) ryanodine (10 μM) depletion of intracellular Ca^{2+} stores, and (2) thapsigargin (THAPS, 2 μM) or cyclopiazonic acid (CPA, 10 μM) depletion of intracellular Ca^{2+} stores on HPV in canine pulmonary artery.

2 Isometric tension was measured from arterial ring suspended in Krebs-Henseliet solution (K-H) bubbled with 95%O_2/5%CO_2. Hypoxia was induced by bubbling phenylephrine (PE, 1 μM) precontracted rings with 95%N_2/5%CO_2. HPV was observed in both intact and endothelial-denuded arteries and expressed as % of maximal KCl contraction (%T_{kmax}) = 21.3 ± 3.2%; n = 13 and 21.7 ± 4%; n = 4, respectively.

3 When SR caffeine sensitive Ca^{2+} stores were depleted by pretreatment with ryanodine and brief caffeine (15 mM) exposure, the hypoxic response was signifcantly reduced to 19.1 ± 9.2% of the control hypoxic contraction (n = 7; P < 0.001) with little or no effect on PE or KCl contractions. On the other hand, in normoxic rings pretreated with THAPS or CPA, the PE responses were significantly reduced (%T_{kmax} = 18.2 ± 3.1% compared to 39.0 ± 3.9% in control; n = 16; P < 0.001; %T_{kmax} = 3.4 ± 1.6% compared to 49.9 ± 7.9% in control; n = 6; P < 0.001; respectively) with no significant effect on caffeine-induced contractions, suggesting that both THAPS and CPA preferentially deplete $InsP_3$-sensitive Ca^{2+} stores, without affecting the caffeine-sensitive Ca^{2+} store; consistent with the existence of separate and independent $InsP_3$ and caffeine-sensitive Ca^{2+} stores in this preparation.

4 When hypoxia was induced in the presence of THAPS or CPA, developed tension was significantly larger than control (%T_{kmax} = 64.5 ± 6.0%; n = 16; P < 0.05%; %T_{kmax} = 78.2 ± 15%; n = 6; P < 0.05; respectively), was partially blocked by nisoldipine (10 μM) and ryanodine (%T_{kmax} = 20.3 ± 3.7%; n = 6), and nearly completely blocked by SK&F 96365 (50 μM). However, the actions of SK&F 96365 appeared to be nonselective since this compound also significantly reduced contractions elicited by KCl, PE and caffeine.

5 Finally, evidence was obtained suggesting: (a) that at least some of the Ca^{2+} released from the caffeine- and ryanodine-sensitive Ca^{2+} stores by hypoxia may be taken up and buffered by the $InsP_3$-sensitive Ca^{2+} stores, and (b) the apparent dependence of HPV on extracellular Ca^{2+} entry pathways may be partially due to the dependence of the Ca^{2+} content of intracellular SR Ca^{2+} stores on sarcolemmal Ca^{2+} entry pathways.

6 These data suggest that caffeine- and ryanodine-sensitive SR Ca^{2+} stores contribute significantly to HPV under normal conditions and, in the presence of THAPS or CPA, an additional nisoldipine- and ryanodine-insensitive Ca^{2+} entry pathway is evoked by hypoxia.

Keywords: Hypoxia; pulmonary artery; vasoconstriction; intracellular Ca^{2+} stores

Introduction

Although the phenomena of hypoxic pulmonary vasoconstriction (HPV) has been recognized for over 50 years (Von Euler & Liljestrand, 1946), its mechanism remains largely unknown. Controversy surrounds many of the key aspects of the response. The role of the endothelium and endothelial-derived mediators remains unresolved, with studies showing that HPV is completely endothelium-dependent, partially endothelium-dependent, or endothelium-independent (Holden & McCall, 1984; Burke-Wolin & Wolin, 1989; Demiryurek *et al*., 1991; Yang & Mehta, 1995). While some of these differences may be related to species differences or size of the arterial vessel (Madden *et al*., 1985; Shirai *et al*., 1986), the demonstration of HPV in enzymatically dispersed pulmonary arterial smooth muscle cells (Madden *et al*., 1992) suggests that at least part of the mechanism for HPV may be intrinsic to the pulmonary smooth muscle cells themselves.

The role played by agonists in HPV has long been recognized but their exact function and mechanism of action remains unknown. In most, but not all species, agonist precontraction is necessary to augment or unmask the contractile response to hypoxia in isolated pulmonary arteries (Madden *et al*., 1985; Rodman *et al*., 1989; Ogata *et al*., 1992; Hoshino *et al*., 1994). However, this dependence on agonist priming may only be apparent under *in vitro* conditions, since there is expected to be some basal agonist tone under *in vivo* conditions.

An essential role of intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, in HPV has been well established. A reduction of P_{O_2} causes membrane depolarization (Harder *et al*., 1985) and vasoconstriction which is sensitive to organic Ca^{2+} channel antagonists (McMurtry *et al*., 1976; Harder *et al*., 1985; Archer *et al*., 1985) suggesting an important role for depolarization induced Ca^{2+} entry. Recent studies have demonstrated that hypoxia inhibits voltage-dependent K^+ currents (K_v) causing depolarization of the resting membrane potential in both acutely isolated and cultured pulmonary arterial smooth

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muscle cells (Post *et al*., 1992; Yuan *et al*., 1993) and chronic hypoxia is associated with a reduction in K_v currents in rat pulmonary artery (Smirnov *et al*., 1994). These studies, therefore, suggest that K^+ channel inhibition may be a critical early event in the initiation of HPV. However, the role of intracellular Ca^{2+} release in HPV is less clear. It has been suggested that hypoxia causes mobilization of Ca^{2+} from intracellular Ca^{2+} stores and this may represent an important early trigger for HPV in some tissues (Harder *et al*., 1985; Hoshino *et al*., 1988; Vadula *et al*., 1993; Salvaterra & Goldman, 1993). It is also conceivable that the sarcoplasmic reticulum of pulmonary arterial smooth muscle cells may act as a superficial Ca^{2+} buffer (van Breemen & Saida, 1989; van Breemen *et al*., 1995) which could play a role in HPV. Finally, there have been suggestions that Ca^{2+} sensitization of the contractile proteins may also play an essential role in HPV in some tissues (Jin *et al*., 1992; Robertson *et al*., 1995).

Our previous electrophysiological studies of canine enzymatically dispersed isolated pulmonary arterial smooth muscle cells suggested that a reduction of P_{O_2} may elicit an early release of Ca^{2+} from intracellular stores, which may cause inhibition of a delayed rectifier type of K^+ channel (Gelband *et al*., 1993), possibly leading to membrane depolarization and subsequent entry of Ca^{2+} through voltage-dependent Ca^{2+} channels (Post *et al*., 1992; 1995). Few contractile studies in canine intact pulmonary arteries have been performed (Hoshino *et al*., 1994), and the role of intracellular Ca^{2+} stores in the contractile response of this tissue to hypoxia has not been previously assessed. The purpose of the present experiments was to examine the role of intracellular Ca^{2+} stores in the hypoxic contraction of canine intact pulmonary arterial rings. Preliminary accounts of these results have been published (Gelband *et al*., 1995; Jabr & Hume, 1997).

Methods

Mongrel dogs of either sex (15–25 kg) were killed with an overdose of pentobarbitone sodium (45 mg kg^{-1}, i.v.) and ketamine (15 mg kg^{-1}, i.v.). The lungs and heart were excised *en bloc* and 3rd and 4th branches ($\approx$700 μm outer diameter) of the pulmonary artery were removed. The vessels were cleaned of all connective tissue and rings were cut ($\approx$4–5 mm) and placed in cold standard Krebs-Henseleit (K-H) solution of the following composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and D-glucose 5.5.

Arterial rings were then suspended in organ chambers (10 ml) maintained at 37°C and perfused with normoxic gas mixture (95%/5% O_2/CO_2; pH = 7.4 $\pm$ 0.04). The rings were mounted on two tungsten triangles suspended between stainless steel wire hooks, one of which was anchored to the organ bath, and the other was connected to a force transducer (grass model FTO3). Tension was continuously recorded and digitized on-line with a MP100WS data acquisition and analysis system (Biopac Systems, Inc., Goleta, CA, U.S.A.) and an IBM compatible 486 computer.

Before the start of each experiment, arterial rings were allowed to equilibrate for 60 min during which the tissues were washed with fresh standard K-H solution at 10–15 min intervals. Also during the equilibration period, a resting tension of 0.75 g was placed on the rings. (This load was determined during preliminary experiments (n = 9) to be optimal resting tension for maximal active tension development in response to high K^+ for these preparations (data not shown). After this initial equilibration period, the viability of the tissues was tested by recording the response to a high K^+ K-H solution. This was composed of the standard K-H solution with KCl increased to 60 mM and a compensatory decrease in NaCl to maintain osmolarity. KCl 60 mM was determined in preliminary dose-response experiments (n = 12) to be the lowest concentration to develop maximal tension in this tissue (data not shown). Subsequently, all other contractions were expressed as a percentage of this maximal KCl contraction (T_{kmax}) in each individual arterial ring thus allowing each tissue to be its own control. Most experiments were performed on rings with an intact functional endothelium. However, some tissues were mechanically denuded of endothelium. The presence or absence of a functional endothelium was confirmed by determining the relaxant response to acetylcholine (ACH; 10^{-7}, 10^{-6} and 10^{-5} M) in rings precontracted with phenylephrine (PE; 10^{-6} M). Relaxation to ACH was observed only in arterial rings with functional endothelium (Furchgott & Zawadzki, 1980).

In experiments where the effect of hypoxia on pulmonary artery rings was determined, arterial rings were usually preconstricted first with PE in standard K-H solution. Then hypoxia (HYP) was induced by changing the gas mixture perfusing the chambers from normoxic (95% O_2/5% CO_2) to hypoxic gas mixture (95% N_2/5% CO_2). The P_{O_2} was determined in preliminary experiments with an O_2 sensitive electrode (MI-730; Microelectrodes, Inc., Londonderry, NH, U.S.A.) to be 692 $\pm$ 0.6 mmHg during normoxic gas perfusion and fell to 17.7 $\pm$ 2.3 mmHg within 5 min of hypoxic exposure. The pH remained at 7.4 $\pm$ 0.04. When the hypoxic-induced contraction reached a plateau (10–15 min), arterial rings were then re-oxygenated with normoxic gas and washed with standard K-H solution and left to equilibrate for 40–50 min. During this period, arterial rings were exposed to 60 mM K^+ K-H solution to ensure their continued viability. All data are expressed as a percentage of the maximum K^+ contraction (T_{kmax}).

Chemicals and drugs

SK&F 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl}-1H-imidazole) hydrochloride was obtained from Calbiochem (La Jolla, CA, U.S.A.) and nisoldipine was kindly provided by Miles Inc. (West Haven, CT, U.S.A.). Ryanodine was obtained from Agrisystems International (Windgap, PA, U.S.A.). All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

All data are presented as mean $\pm$ s.e. Statistical significance difference in the same group was determined by two-tailed Student's t test. Whereas significant comparison between multiple groups were performed by one-way analysis of variance (ANOVA). A P value <0.05 was accepted as statistically significant.

Results

Dependence of hypoxic response on agonist pretreatment

Since agonist preconstriction of tissue is often a necessary prerequisite to demonstrate hypoxic pulmonary vasoconstriction (HPV) *in vitro*, a dose-response curve for phenylephrine (PE) was first established with concentrations from 10^{-9} M through to 10^{-3} M. Activation of α_1-adrenoceptors by PE in smooth muscle is known to result in hydrolysis of inositol phospholipids into inositol 1,4,5-trisphosphate ($InsP_3$) which causes Ca^{2+} release from intracellular Ca^{2+} stores [Ca^{2+}], and diacylglyerol (DAG), which causes Ca^{2+} sensitization through protein kinase C (PKC) activation (Somlyo & Somlyo, 1994). Concentrations of PE ([PE]) produced threshold increases in tension in the range of 10^{-8} M, which saturated near 100% of T_{kmax} at concentrations near 10^{-4} M (Figure 1a). The resulting dose-response curve for PE (Figure 1b) was constructed from responses in a total of 12 pulmonary arterial rings and was fit to a simple binding equation (solid line), with an estimated $K_d = 3 \times 10^{-7}$ M.

PE concentrations of 0, 10^{-7}, 10^{-6} and 10^{-5} M were chosen to examine the dependence of the hypoxic contractile response on agonist concentration since these concentrations resulted in

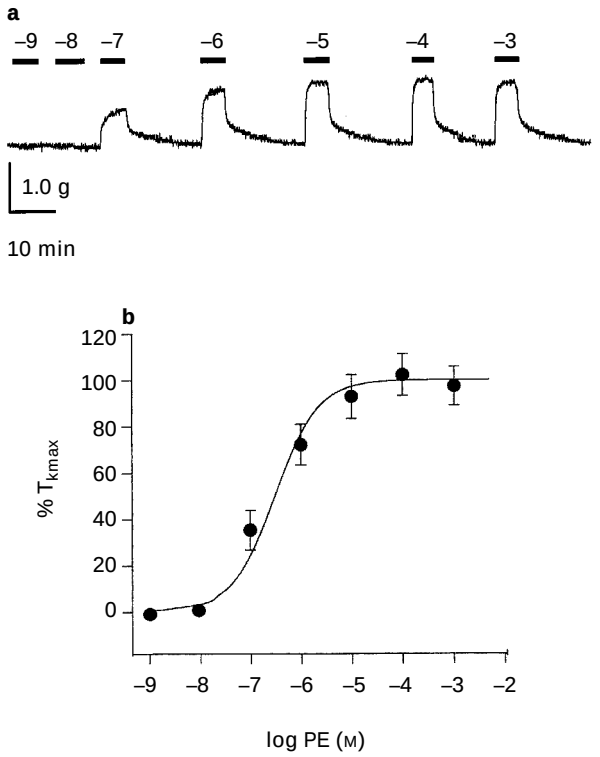

Figure 1 Effect of different concentrations of phenylephrine (PE) on pulmonary arterial ring contraction. (a) Representative recordings of isometric tension obtained from arterial ring at [PE] of 10^{-9} to 10^{-3} M. (b) Dose-response curve for PE-induced contraction fitted to a simple binding equation, estimated $K_d = 3 \times 10^{-7}$ M. Values are means of % T_{kmax} ($n = 12$); vertical lines show s.e.mean.

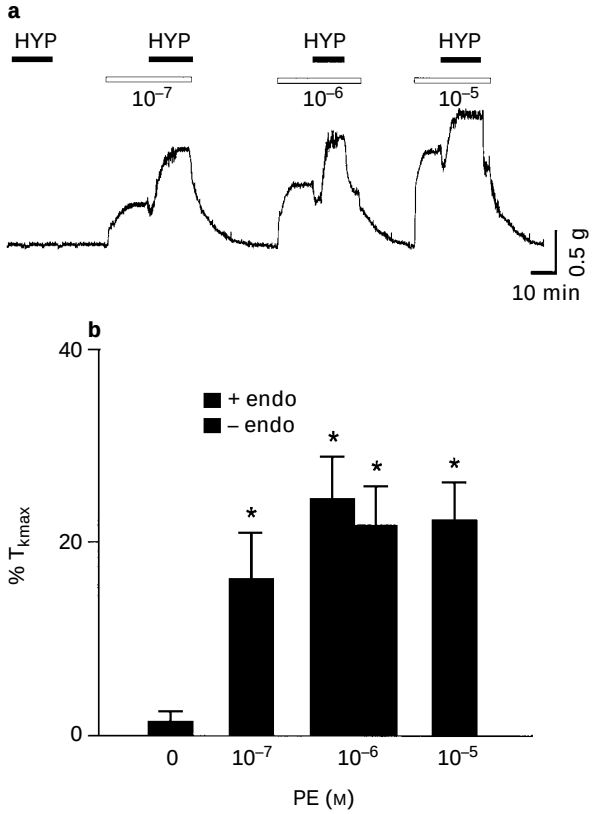

Figure 2 Effect of different concentrations of phenylephrine (PE) on pulmonary arterial ring contraction in response to hypoxia (HYP). (a) Representative recording of isometric tension during hypoxia following preconstriction with different [PE] ranging from 10^{-9} to 10^{-3} M. (b) Mean of hypoxic contractions developed at different [PE]. Data were obtained from endothelium-intact (+endo; $n = 12$) and endothelium-denuded (−endo; $n = 6$) rings, respectively. Each column represents mean of % $T_{kmax} \pm$ s.e.

a wide range of tension development (0%, $36.1 \pm 8.5\%$, $72.8 \pm 8.7\%$, $93.4 \pm 9.5\%$ of T_{kmax}). Pulmonary arterial rings were each exposed to different [PE] and the effects of hypoxia were examined. The response of each ring was directly compared to its T_{kmax} to reduce variability and to allow each tissue to act as its own control. As shown in Figure 2a, in the absence of PE, hypoxia failed to elicit any significant contraction. However, tissues preconstricted with PE exhibited a biphasic contractile response to hypoxia. There was an initial small transient relaxation followed by a sustained increase in tension, which was reversed upon reoxygenation and washout of PE. The dependence of the sustained phase of hypoxic contraction on PE concentration from 14 pulmonary rings are summarized in Figure 2b. No significant differences were observed between hypoxic contractions elicited in the presence of 10^{-7}, 10^{-6} or 10^{-5} M PE ($16.5 \pm 3.4\%$, $23.6 \pm 3.4\%$, $21.2 \pm 3.5\%$ of T_{kmax}, respectively). However, the dependence of HPV on agonistic priming was shown by the significant lack of hypoxic contraction in the absence of PE. Since no significant difference was shown for HPV with different levels of agonist exposure, all further hypoxic contractions were induced with 1 μM PE. When precontracted with 1 μM PE, arterial rings showed an average increase in tension of $47.5 \pm 5.0\%$ of T_{kmax} and the mean increase in tension during hypoxia was $23.6 \pm 3.4\%$ of the T_{kmax} ($n = 14$). We also confirmed that HPV did not require the presence of a functional endothelium in this tissue (Hoshino *et al*., 1994), since in six rings denuded of the endothelium, in the presence of 1 μM PE, hypoxia produced a mean increase in tension of $21.8 \pm 4.0\%$ of T_{kmax} (Figure 2b).

Effects of ryanodine on HYP contractions

To examine whether or not Ca^{2+} release from intracellular stores may play a role in the hypoxic contraction, rings ($n = 18$) were pretreated with ryanodine (10 μM), an agent well known to cause release of intracellular Ca^{2+} and eventual depletion of the SR Ca^{2+} store in a variety of muscle cells including smooth muscle (Hwang & van Breeman, 1987; Wagner-Mann *et al*., 1992). In each ring, the effects of KCl, PE and HYP were first examined in the absence of ryanodine, and then tissues were exposed to ryanodine for about 40 min, and then the effects of KCl, PE and hypoxia (HYP) were reexamined in the continued presence of ryanodine. As illustrated in Figure 3a and b, ryanodine pretreatment had no significant effect on KCl (60 mM) contractions or PE (1 μM) contractions ($42.5 \pm 4.3\%$ and $45.6 \pm 3.7\%$ of T_{kmax}, respectively; $n = 18$). However, 10 μM ryanodine caused a $27.6 \pm 5.5\%$ of T_{kmax} decrease in the sustained contractile response to hypoxia. Since ryanodine had little effect on the contractile response to PE, these results suggest that in canine pulmonary artery, $InsP_3$-sensitive Ca^{2+} stores are probably independent of the ryanodine-sensitive Ca^{2+} store. The small effect of ryanodine on T_{kmax} indicates that KCl induces some small activation of intracellular Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. However, the most significant effect of ryanodine was on the hypoxic contractions indicating that at least part of the hypoxic contractile response in this tissue may involve intracellular Ca^{2+} release from a ryanodine-sensitive Ca^{2+} store. However, in these experiments it is not clear to what extent ryanodine pretreatment depleted the ryanodine-sensitive Ca^{2+} store. If this store is only partially depleted, then this could lead to an underestimation of the role of intracellular Ca^{2+} release from this store in HPV. To assess the degree of Ca^{2+} depletion by ryanodine, a separate group of tissues were exposed to ryanodine under identical experimental conditions and the response to caffeine was determined and compared to caffeine responses from tissues

not exposed to ryanodine; 40 min exposure to 10 μM ryanodine reduced the caffeine contraction by an average of only $18.5 \pm 13.7\%$ ($n=6$) compared to controls.

In order to ensure more complete depletion of the ryanodine-sensitive Ca^{2+} store under these conditions, we repeated these experiments in the presence of the same concentration of ryanodine for 60 min, followed by two brief (2 min) exposures to caffeine (15 mM). We have used this method previously to produce effective depletion of ryanodine-sensitive Ca^{2+} stores (Ishikawa *et al*., 1993). When tissues were pretreated with ryanodine with two brief caffeine exposures, the second caffeine contractile response was a small relaxation ($4.9 \pm 2.0\%$ of control; $n=7$, $P<0.001$), indicating nearly complete depletion of the store (Figure 3c). Following washout of caffeine, in the continued presence of ryanodine, the hypoxic contractions in the presence of 1 μM PE were reduced to $19.1 \pm 9.2\%$ ($n=7$; $P<0.001$) of the control hypoxic contraction. Little change was observed in the KCl or PE contractions. Thus, ryanodine pretreatment with a brief caffeine exposure caused a signficantly greater attenuation of the hypoxic contraction, compared to ryanodine pretreatment alone ($P<0.05$), suggesting that Ca^{2+} release from the ryanodine- and caffeine-sensitive Ca^{2+} store in this tissue plays a prominent role in the hypoxic contraction.

Effects of thapsigargin and cyclopiazonic acid

To examine the role of intracellular Ca^{2+} release in the hypoxic contraction further, we also tested the effects of thapsigargin (THAPS) and cyclopiazonic acid (CPA) on hypoxic pulmonary contractions. These agents are believed to be selective inhibitors of the SR Ca^{2+}-ATPase (Seidler *et al*., 1989; Thastrup *et al*., 1990; Kirby *et al*., 1992) and have been shown to prevent Ca^{2+} accumulation and refilling of the SR Ca^{2+} stores in rabbit vena cava smooth muscle (Chen & van Breemen, 1993) and rat pulmonary artery (De La Fuente *et al*., 1995). If THAPS and CPA are effective in causing depletion of both $InsP_3$- and ryanodine-sensitive Ca^{2+} stores, we expected that the effects of these agents on hypoxic contraction might be similar to those of ryanodine.

As shown in Figure 4, in the presence of 2 μM THAPS, there was a much smaller PE-induced contraction compared to control, but surprizingly the HYP-induced contraction in the presence of PE was much larger than that observed in the control. The results from a number of experiments are summarized in Figure 4b. THAPS failed to alter T_{kmax} signficantly ($94.03 \pm 3.3\%$, $n=16$), but produced a significant reduction in the PE-induced contraction ($18.2 \pm 3.1\%$ of T_{kmax}, $n=16$), compared to control ($39.0 \pm 3.9\%$ of T_{kmax}, $n=16$; $P<0.05$), and the HYP-induced contraction was significantly potenti-

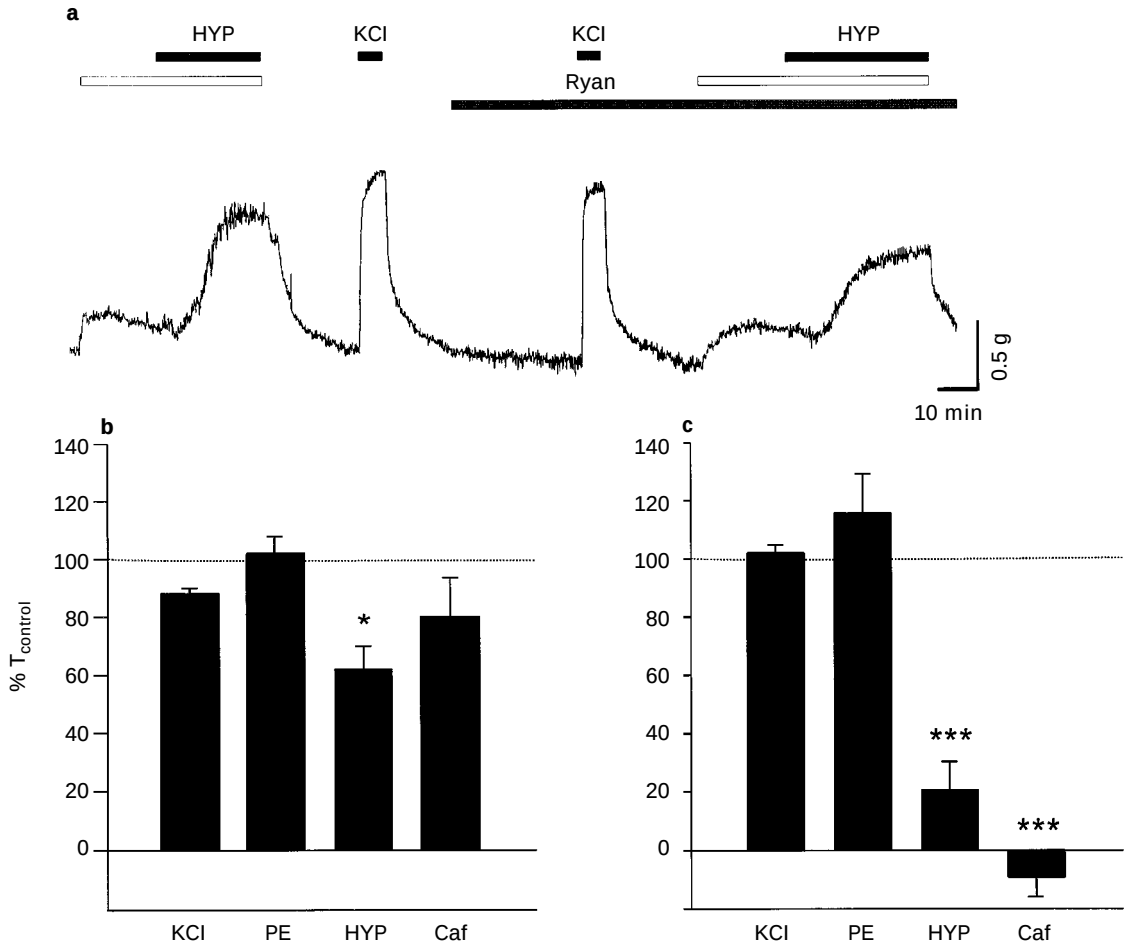

Figure 3 Effects of depletion of ryanodine-sensitive Ca^{2+} stores on pulmonary arterial ring contraction response to hypoxia. (a) Representative recording of isometric tension developed with 60 mM KCl, and during hypoxia (HYP) following PE (1 μM) preconstriction (open bar) before and after pretreatment with 10 μM ryanodine (Ryan) for 40 min. (b) Mean of PE- and hypoxic-induced contractions following ryanodine pretreatment alone ($n=18$); 2 min application of caffeine (Caf; 15 mM) following a similar exposure to ryanodine was used to test for store depletion. (c) Mean of PE- and hypoxic-induced contractions after complete depletion of ryanodine-sensitive $[Ca^{2+}]_i$ store by 60 min pretreatment with 10 μM ryanodine and a 2 min exposure to 15 mM caffeine ($n=7$; see Results). A second 2 min exposure to caffeine was used to test for store depletion. Each column represents mean of % control $\pm$ s.e. $^*P<0.05$, $^{***}P<0.001$. The absolute change in tension (compared to the baseline before PE exposure) during hypoxia was $74.1 \pm 5.2\%$ T_{kmax} in the control, $67.4 \pm 4.8\%$ T_{kmax} in the presence of ryanodine alone, and $51.7 \pm 2.8\%$ T_{kmax} in the presence of ryanodine, following the brief caffeine exposure.

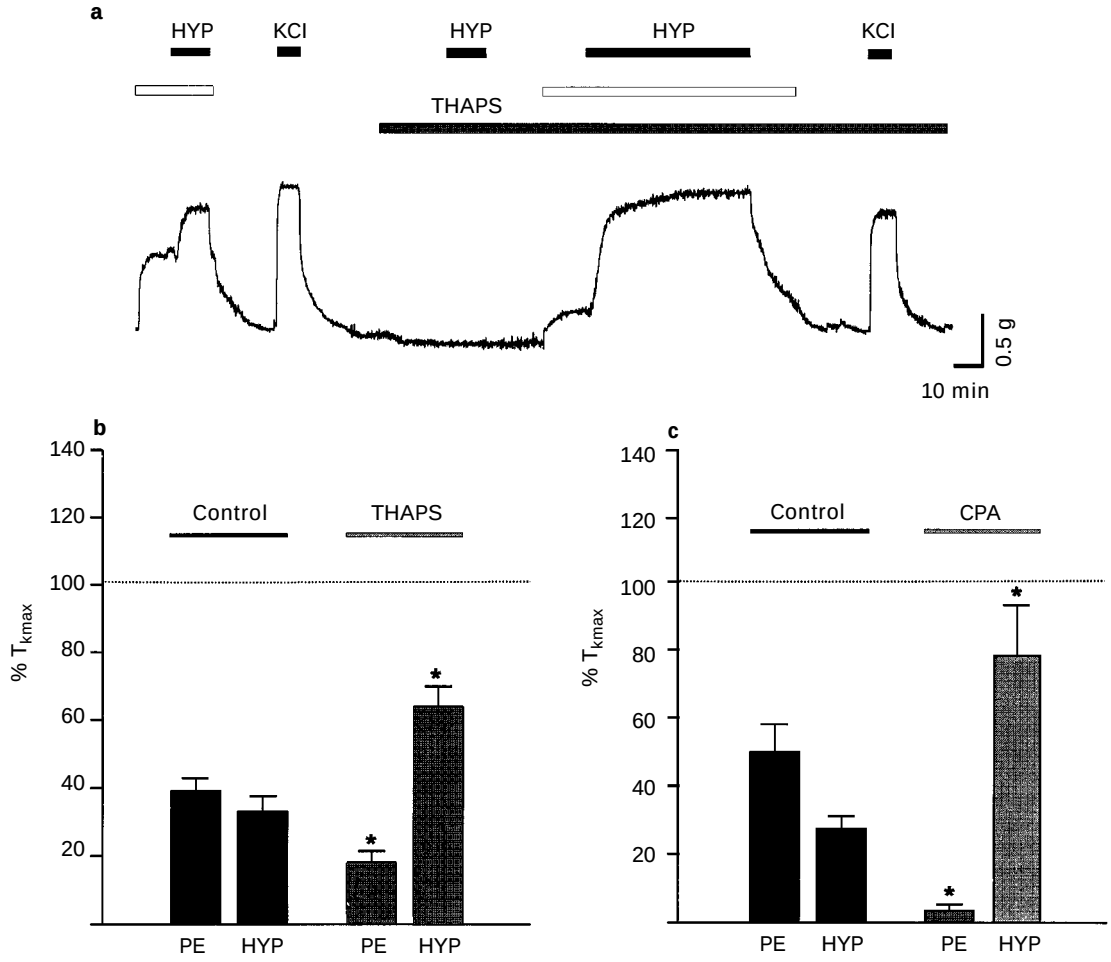

Figure 4 Effects of depletion of $InsP_3$-sensitive Ca^{2+} stores with thapsigargin (THAPS) and cyclopiazonic acid (CPA) on pulmonary arterial ring contractions in response to PE and hypoxia. (a) Representative recording of isometric tension developed with 60 mM KCl and during hypoxia following 1 μM PE preconstriction (open bar) in the absence and presence of 2 μM THAPS. (b) PE- and hypoxic-induced contraction in the absence ($n=16$) and presence of 2 μM THAPS ($n=16$). (c) PE- and hypoxic-induced contractions in the absence ($n=6$) and presence of 10 μM CPA ($n=6$). Each column represents mean of % $T_{kmax} \pm$s.e. $^*P<0.05$, significant difference between treated groups and its corresponding control.

ated ($64.5 \pm 6.0\%$ of T_{kmax}, $n=16$; $P<0.05$), compared to control ($32.9 \pm 4.4\%$ of T_{kmax}, $n=16$). Nearly identical results were obtained with CPA (Figure 4c). 10 μM CPA failed to alter T_{kmax} significantly ($92.5 \pm 19.4\%$ of control, $n=6$), but produced a significant reduction in the PE-induced contraction ($3.4 \pm 1.6\%$ of T_{kmax}, $n=6$; $P<0.05$), compared to control ($49.9 \pm 7.9\%$ of T_{kmax}, $n=6$), and the HYP-induced contraction was significantly potentiated ($78.2 \pm 15.0\%$ of T_{kmax}, $n=6$; $P<0.05$), compared to control ($27.3 \pm 3.4\%$ of T_{kmax}, $n=6$). Neither THAPS nor CPA alone caused any significant increase in resting tone in these experiments.

The significant and large reduction in the amplitude of PE contractions by both THAPS and CPA are consistent with depletion of $InsP_3$-sensitive Ca^{2+} stores and suggest that the major contractile effects of PE can be attributed to release of Ca^{2+} from these stores. However, the observed potentiation of the HYP contraction by these agents was unexpected and not consistent with the expected effects of depletion of both the caffeine- and ryanodine-sensitive Ca^{2+} store on the HYP contraction (cf. Figure 3). The fact that ryanodine with caffeine exposure nearly eliminated any subsequent response to caffeine and significantly reduced the HYP contraction, while having little or no effect on the PE contraction, is evidence that $InsP_3$- and ryanodine-sensitive Ca^{2+} stores in canine pulmonary artery may be completely separate and independent. It is possible that THAPS and CPA may only deplete the $InsP_3$-sensitive Ca^{2+} store in canine pulmonary artery, and have little or no effect on the caffeine- and ryanodine-sensitive Ca^{2+} store. In order to test this possibility, we examined the effects of CPA on the caffeine-sensitive Ca^{2+} store directly by determining the effects of CPA on caffeine-induced contractions. Under control conditions prior to exposure to CPA, brief exposure to 15 mM caffeine produced contractions which were $29.9 \pm 3.9\%$ of T_{kmax} ($n=7$). In the presence of 10 μM CPA, 15 mM caffeine-induced contractions were nearly identical to those observed in the control, $33.3 \pm 2.8\%$ of T_{kmax} ($n=7$). These data, therefore, suggest that THAPS and CPA preferentially deplete $InsP_3$-sensitive Ca^{2+} stores, and have little effect on caffeine- and ryanodine-sensitive Ca^{2+} stores in canine pulmonary artery. This may partly explain why these agents fail to reduce signficantly the amplitude of HYP contractions in this tissue, since these seem to be largely dependent upon the presence of a functional caffeine- and ryanodine-sensitive Ca^{2+} store under normal conditions.

Interestingly, CPA did alter the response to ryanodine. In control tissues, ryanodine alone or following a brief caffeine contraction, failed to produce any noticeable effect on resting tension (top trace, Figure 5). However, in CPA-treated tissues, there was consistently a small sustained increase in resting tension following a brief caffeine contraction in the continued presence of ryanodine. In 6 tissues, the sustained increase in resting tension was $8.5 \pm 1.4\%$ of T_{kmax}. This observation is consistent with the possibility that under normal conditions, some of the Ca^{2+} released from caffeine- and ryanodine-sensitive Ca^{2+} stores may be taken up and buffered by the $InsP_3$ sensitive Ca^{2+} stores.

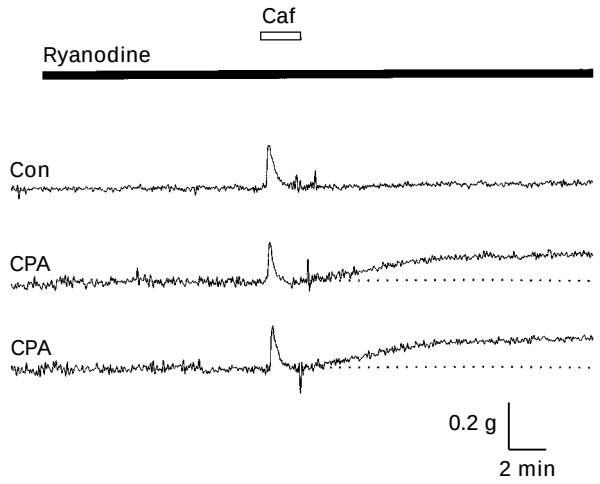

Figure 5 Effects of ryanodine alone and following a brief caffeine (Caf) contraction on resting tension in control (Con) or cyclopiazonic acid (CPA)-treated tissues. Tissues were exposed to 10 μM ryanodine and subsequently to a 2 min application of caffeine (15 mM). Tissues shown in the bottom two traces were exposed to 10 μM CPA for at least 20 min before ryanodine exposure.

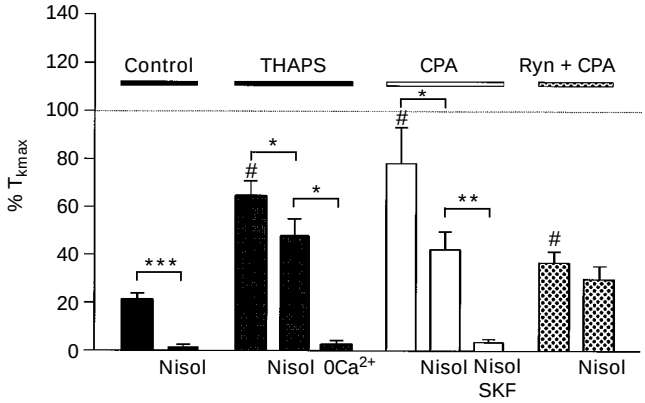

Figure 6 Source of Ca^{2+} mediating hypoxic-induced contractions in the absence and presence of thapsigargin (THAPS) or cyclopiazonic acid (CPA). Summary of data representing: mean of hypoxic-induced contractions under control conditions and after addition of 10 μM nisoldipine (NISOL); ($n=4$); mean of hypoxic contraction in the presence of 2 μM THAPS and the effects of 10 μM nisoldipine ($n=7$), or nominally Ca^{2+} free on such contractions ($n=5$), mean of hypoxic-induced contractions in presence of 10 μM CPA and the effects of 10 μM nisoldipine and 50 μM SK&F 96365 ($n=6$), and mean of hypoxic-induced contractions in presence of 10 μM CPA following complete depletion of ryanodine-sensitive $[Ca^{2+}]_i$ stores (10 μM ryanodine for 40 min with 2 min application of 15 mM caffeine before and after superfusion with 10 μM nisoldipine ($n=6$). Each column represents mean of % $T_{kmax} \pm$ s.e. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, significant difference between each treated group difference with respect to its corresponding control by Student's t test. $\#P<0.05$ significant between different groups and the control group by ANOVA.

Source of Ca^{2+} for HYP contractions in the absence and presence of THAPS and CPA

The role of Ca^{2+} entry through voltage-dependent Ca^{2+} channels in HPV in canine pulmonary artery is not clear, given the results obtained with ryanodine suggesting a prominent role of intracellular Ca^{2+} release in HYP contractions. Therefore, we evaluated the role of sarcolemmal Ca^{2+} entry pathways on HYP contractions by examining the effects of nisoldipine, an organic Ca^{2+} channel antagonist, and superfusion with nominally Ca^{2+} free external solutions on HYP pulmonary contractions under normal conditions and in the presence of CPA.

As shown in Figure 6, 10 μM nisoldipine nearly completely blocked HYP contractions under control conditions. Before nisoldipine, HYP contractions were $23.0 \pm 3.7\%$ of T_{kmax} ($n=4$), whereas in the presence of nisoldipine, HYP contractions were reduced to $1.5 \pm 1.3\%$ of T_{kmax} ($n=4$; $P<0.001$). These data suggest that Ca^{2+} entry involving predominantly L-type Ca^{2+} channels provides an important source of Ca^{2+} for HYP contractions under normal conditions. However, in THAPS or CPA treated tissues, the enhanced HYP contractions were only partially reduced by 10 μM nisoldipine, even though in THAPS the enhanced HYP contraction was nearly completely blocked by superfusion with Ca^{2+} free solution. This suggests that the enhanced HYP contractions observed in THAPS or CPA treated tissues involves both nisoldipine-sensitive and insensitive Ca^{2+} entry pathways, in contrast to control HYP contractions, which appear to involve only a nisoldipine-sensitive Ca^{2+} entry pathway. This conclusion was further supported by the observations that the nisoldipine-insensitive Ca^{2+} pathway for HYP contractions in CPA, were not affected by ryanodine pretreatment (with caffeine) but were nearly completely inhibited by the compound, SK&F 96365 (50 μM), a putative inhibitor of a Ca^{2+}-store depletion-induced Ca^{2+} entry pathway in some tissues (Chung *et al*., 1994; Koch *et al*., 1994).

While the source of Ca^{2+} for normal HYP contractions appears to involve a nisoldipine-sensitive Ca^{2+} entry pathway, the enhanced HYP contractions observed in THAPS or CPA treated tissues appears to involve additionally a novel nisoldipine-insensitive Ca^{2+} entry which is blocked by SK&F 96365. However, the nature of this novel Ca^{2+} entry activated by HYP in THAPS or CPA treated tissues is not very clear, since the specificity of SK&F 96365 is uncertain. Although SK&F 96365 is known to block voltage-dependent Ca^{2+} channels also (Merritt *et al*., 1990), this action is unlikely to be responsible for the inhibition of HYP contractions in CPA treated canine pulmonary arteries, since it was observed to block the nisoldipine-insensitive HYP contractions. In order to define the specificity of action of SK&F 96365 better, we examined the effects of this compound on contractions induced by KCl, PE and caffeine (Figure 7). 50 μM SK&F 96365 reduced KCl contractions to $9.7 \pm 1.5\%$ of T_{kmax} ($n=9$; $P<0.001$), PE contractions from $62.7 \pm 6.3\%$ of T_{kmax} to $10.2 \pm 3.6\%$ ($n=9$; $P<0.001$), and caffeine contractions from $37.2 \pm 2.0\%$ of T_{kmax} to $2.7 \pm 2.9\%$ ($n=9$; $P<0.001$). These

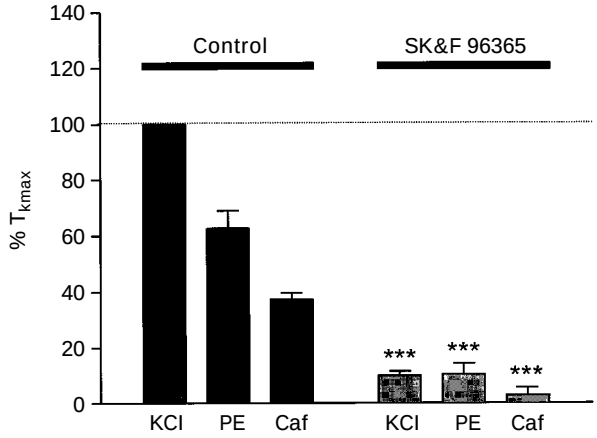

Figure 7 Effects of 50 μM SK&F 96365 on pulmonary arterial ring contraction in response to 60 mM KCl, PE and caffeine (Caf). Columns represent mean of 60 mM KCl, 1 μM PE and 15 mM caffeine-induced contractions in the absence ($n=9$) and presence of 50 μM SK&F 96365 ($n=9$). Each column represents mean of % $T_{kmax} \pm$ s.e. $^{***}P<0.001$, represent a significant difference between SK&F 96365-treated groups and its corresponding controls.

results suggest that SK&F 96365 is a rather nonselective compound, capable of blocking a variety of Ca^{2+} entry and Ca^{2+} release processes.

Sensitivity of HYP contractions to calcium channel antagonists may involve depletion of intracellular Ca^{2+} stores

Our data aappear to provide somewhat conflicting results with regard to the sources of Ca^{2+} required for HYP contractions under normal conditions in canine pulmonary artery. Experiments examining the sensitivity of HYP contractions to ryanodine provided strong evidence suggesting an important role for Ca^{2+} release from distinct caffeine- and ryanodine-sensitive Ca^{2+} stores since HYP contractions were reduced by approximately 80%. Yet the data in Figure 6 strongly suggest the involvement of voltage-dependent Ca^{2+} channels, since HYP contractions are nearly completely eliminated by organic Ca^{2+} channel antagonists. These apparently conflicting results might be resolved if the Ca^{2+} content of intracellular Ca^{2+} stores in canine pulmonary artery is tightly linked to sarcolemmal Ca^{2+} influx. Perhaps, interventions which reduce sarcolemmal Ca^{2+} entry significantly reduce Ca^{2+} content of intracellular Ca^{2+} stores. To test this possibility, we examined the effects of nisoldipine and superfusion with Ca^{2+} free solutions on PE contractions induced by Ca^{2+} release from $InsP_3$ Ca^{2+} stores and caffeine contractions, induced by Ca^{2+} release from separate caffeine- and ryanodine-sensitive Ca^{2+} stores in this tissue.

Figure 8a shows that if PE (1 μM) contractions are used to assess the extent of the Ca^{2+} load in the $InsP_3$ Ca^{2+} store, this store appears to be rapidly depleted within 5 min exposure to nominally Ca^{2+} free external solutions. Exposure to nominally Ca^{2+} free external solution reduced T_{kmax} of PE contractions to $18.8\pm2.1\%$ ($n=5$; $P<0.001$), $11.8\pm1.8\%$ ($n=7$; $P<0.001$), and $8.9\pm3.3\%$ ($n=4$; $P<0.001$) compared to control ($62.9\pm6.1\%$; $n=17$) at 5, 10 and 15 min, respectively. These effects of superfusion with nominally Ca^{2+} free solutions can be attributed primarily to depletion of the $InsP_3$ Ca^{2+} stores, since both THAPS and CPA nearly completely eliminated the PE contractions (Figure 4), suggesting that sarcolemmal Ca^{2+} entry pathways *per se* play little or no role in PE contractions. In contrast, exposure to nisoldipine did not induce any significant change in T_{kmax} of PE contractions, $58.1\pm9.1\%$ ($n=6$), and $51.5\pm4.8\%$ ($n=6$) compared to control ($57.5\pm6.6\%$; $n=12$) at 10 and 15 min, respectively (Figure 8b). These data suggest that a nisoldipine-insensitive Ca^{2+} entry pathway is normally involved in filling $InsP_3$ sensitive Ca^{2+} stores.

When caffeine (15 mM) contractions were used to assess the extent of the Ca^{2+} load in the caffeine- and ryanodine-sensitive Ca^{2+} store, this store also appeared to be rapidly depleted by exposure to nominally Ca^{2+} free external solutions or nisoldipine (Figure 8a). Exposure to nominally Ca^{2+} free external solution significantly reduced T_{kmax} of caffeine contractions to $22.8\pm2.4\%$ ($n=5$; $P<0.001$), $18.8\pm3.1\%$ ($n=7$; $P<0.001$) and $9.6\pm2.6\%$ ($n=4$; $P<0.001$) at 5, 10 and 15 min, respectively, compared to control ($39.4\pm2.8\%$; $n=17$). Exposure to nisoldipine also reduced T_{kmax} of caffeine contractions to $41.6\pm2.4\%$ ($n=5$), and $28.8\pm2.3\%$ ($n=6$; $P<0.001$) at 10 and 15 min, respectively (Figure 7b), compared to control ($50.7\pm3.8\%$; $n=12$). These data suggest that the Ca^{2+} content of caffeine- and ryanodine-sensitive Ca^{2+} stores in canine pulmonary artery is rather tightly linked to sarcolemmal Ca^{2+} influx involving both nisoldipine-sensitive and -insensitive pathways.

Discussion

Although interest in delineating the cellular mechanisms responsible for HPV has increased in recent years, there remain considerable uncertainties with regard to several aspects of the phenomenon and it is not yet even clear which sources of Ca^{2+} normally contribute to the rise in $[Ca^{2+}]_i$ during HPV. Earlier studies which showed that HYP vasoconstriction can be significantly reduced by organic Ca^{2+} channel antagonists (McMurtry *et al*., 1976; Harder *et al*., 1985; Archer *et al*., 1985) suggested an important role for depolarization-induced Ca^{2+} entry through voltage-dependent Ca^{2+} channels, and these observations along with the findings that hypoxia causes membrane depolarization by inhibition of voltage-dependent K^+ channels (K_v) (Post *et al*., 1992; Yuan *et al*., 1993) has led to the hypothesis that direct hypoxic modulation of K^+ channels may be the initial trigger event in HPV (Weir & Archer, 1995; Kozlowski, 1996). How reduced P_{O_2} causes in-

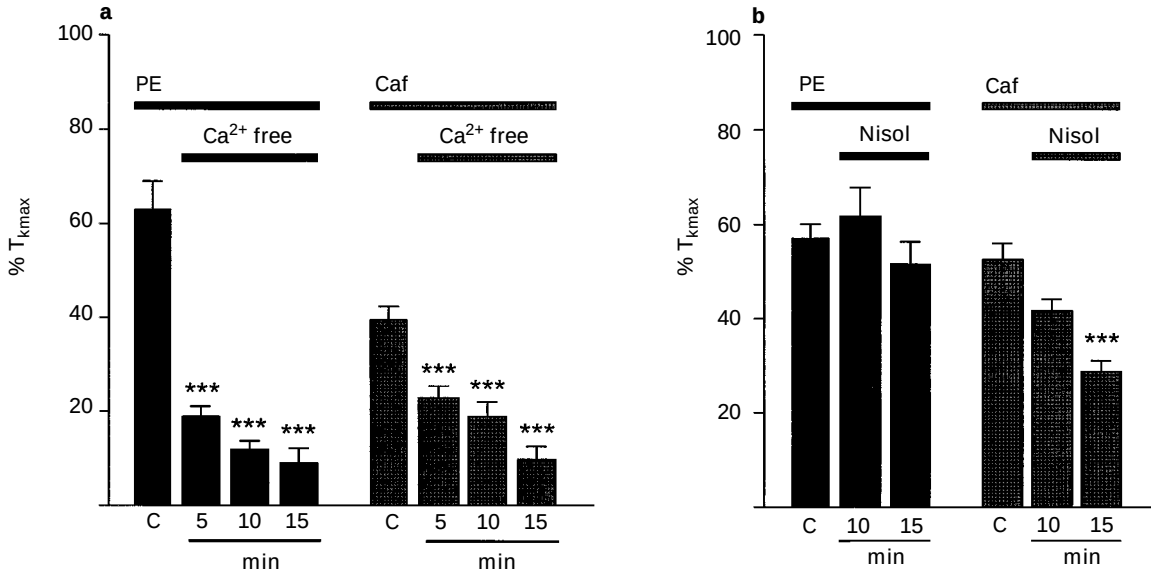

Figure 8 Effect of pretreatment with nominally Ca^{2+} free solutions or nisoldipine (Nisol) on arterial ring vasoconstriction in response to PE and caffeine (Caf). (a) PE-induced contractions in control (C; $n=16$) and after 5 ($n=5$), 10 ($n=7$), and 15 min ($n=4$) incubation with nominally Ca^{2+} free K-H solution, or caffeine-induced contractions in control (C; $n=16$) and after 5 ($n=5$), 10 ($n=7$) and 15 min ($n=4$) incubation in nominally Ca^{2+} free K-H solution. (b) PE-induced contractions in control (C; $n=6$) and after 10 ($n=6$) and 15 min ($n=6$) incubation with 10 μM nisoldipine (Nisol), or caffeine-induced contractions in control (C; $n=6$) before and after 10 ($n=5$) and 15 min ($n=6$) incubation with 10 μM nisoldipine. Each column represents mean of % $T_{kmax}\pm$s.e. ***$P<0.001$, significant difference between treated groups and their corresponding controls.

hibition of K_v channels is not presently well understood, but several possible mechanisms have been proposed, including P_{O_2} regulation of the redox state of K_v channels (Archer *et al*., 1993) and modulation of K_v channels due to alterations in oxidative metabolism (Yuan *et al*., 1996). Yet, this hypothetical mechanism for HPV fails to account for increasing evidence suggesting an important role of release of Ca^{2+} from intracellular Ca^{2+} stores in HPV (Hoshino *et al*., 1988; Vadula *et al*., 1993; Salvaterra & Goldman, 1993). Our previous electrophysiological studies of canine pulmonary arterial cells suggested that HYP inhibition of K_v channels may be indirect and attributed to $[Ca^{2+}]_i$ block of K^+ channels resulting from HYP release of Ca^{2+} from intracellular Ca^{2+} stores (Post *et al*., 1995). The purpose of the present study was, therefore, to examine the possible role of $[Ca^{2+}]_i$ release in the contractile response of canine pulmonary arterial smooth muscle to HYP.

Ryanodine is a rather selective agent which has been used to assess the functional role of intracellular Ca^{2+} stores in a wide variety of different types of cells. Ryanodine reduces the Ca^{2+} handling ability of the caffeine-sensitive SR by locking the Ca^{2+} release channel into a subconductance state, eventually leading to SR Ca^{2+} depletion in a time- and use-dependent manner (Rousseau *et al*., 1987). Ryanodine does not appear to have signficant effects on sarcolemmal Ca^{2+} channels (Hwang & van Breemen, 1987; Balke & Wier, 1991), the plasma membrane Ca^{2+}-ATPase pump (Sutko *et al*., 1985) or $InsP_3$-sensitive Ca^{2+} stores (Iino *et al*., 1988). In our initial experiments, pretreatment of pulmonary arteries with ryanodine produced a modest, but consistent decline (mean 27.7%) in the contractile response to HYP (Figure 3b), suggesting some involvement of a ryanodine sensitive Ca^{2+} store in the HYP contraction. However, it is known that ryanodine is more effective when Ca^{2+} release channels are in an activated state (Rousseau *et al*., 1987), and it has previously been shown in skinned fibres from guinea-pig taenia, portal vein and pulmonary artery that ryanodine pretreatment causes only small depletion of Ca^{2+} in the ryanodine-sensitive Ca^{2+} store, unless Ca^{2+} release channels are also activated by caffeine (Iino *et al*., 1988). Consistent with this observation, we found that ryanodine pretreatment alone caused only a small change in the amplitude of a caffeine contraction. However, following the first caffeine exposure, in the continued presence of ryanodine, a second caffeine exposure failed to elicit any significant contraction (Figure 3c), indicating significant SR Ca^{2+} store depletion. Under this condition, subsequent HYP contractions were reduced by an average of 80.9%. These data strongly suggest that under normal conditions the HYP contraction is largely dependent upon the presence of a functional caffeine- and ryanodine-sensitive SR Ca^{2+} store.

HPV is usually monophasic in whole animal or blood-perfused lungs, but biphasic responses to hypoxia have been observed in isolated rat (Bennie *et al*., 1991; Leach *et al*., 1994) and porcine pulmonary arteries (Kovirtz *et al*., 1993). Although evidence suggests that in some of these tissues the initial transient phase may be due to release of Ca^{2+} from intracellular stores, whereas the second sustained phase may be associated with Ca^{2+} influx through Ca^{2+} channels, there remains uncertainty regarding the actual relationship between these two phases of contraction to HPV (Kozlowski, 1996). It has also been suggested that the second sustained phase is endothelium-dependent and may involve a PKC-independent Ca^{2+} sensitization process (Robertson *et al*., 1995). In contrast, in canine pulmonary arteries, we only observed a sustained monophasic contractile response to hypoxia. Even with longer exposures to hypoxia ($\sim$30 min, Figure 3) only a sustained contractile response was observed. The reasons for these differences are not immediately apparent, but might be due to relative differences in the role of endothelium-derived factors in the hypoxic response or differences in the relationship between the two types of Ca^{2+} stores in different arteries. The observed dependence of the monphasic hypoxic response on functional caffeine- and ryanodine-sensitive SR Ca^{2+} stores in canine pulmonary arteries is similar to that shown for the initial transient contractile response to hypoxia observed in some isolated arteries.

Although we conclude that HYP-induced release of Ca^{2+} from caffeine- and ryanodine-sensitive SR Ca^{2+} stores represents a major mechanism responsible for triggering the HYP contraction in canine pulmonary artery, what is not known is whether or not the SR Ca^{2+} that is released in response to HYP provides the major source of Ca^{2+} to activate the HYP contraction, or whether the released Ca^{2+} also stimulates further Ca^{2+} entry into the cell by affecting sarcolemmal Ca^{2+} entry pathways. For example, membrane depolarization caused by Ca^{2+} inhibition of voltage-dependent K^+ channels (Post *et al*., 1995) or by activation of Ca^{2+}-dependent chloride channels (Clapp *et al*., 1996) might also result in augmentation of Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Indeed, the ability of organic Ca^{2+} channel antagonists to attenuate HYP contractions (Figure 5; McMurtry *et al*., 1976; Harder *et al*., 1985; Archer *et al*., 1985) seems to suggest some role for sarcolemmal Ca^{2+} entry. However, such a role may have previously been overemphasized since it seems clear from the data shown in Figure 8 that the Ca^{2+} content of the SR Ca^{2+} stores appears to be tightly linked to sarcolemmal Ca^{2+} influx in canine pulmonary artery, and interventions which interfere with the latter will inevitably affect the Ca^{2+} content in the SR. As a result, caution should be exercised when experimental interventions are used to assess the role of Ca^{2+} influx to contractile events in some types of smooth muscle, since it seems clear that many of these can also lead to depletion of intracellular Ca^{2+} stores, thus altering processes dependent upon intracellular Ca^{2+} release.

Our data provide some insight into the nature of the SR Ca^{2+} stores in canine pulmonary arterial smooth muscle. Ryanodine pretreatment with or without brief caffeine exposures, failed to alter significantly the contractile response to KCl or PE, suggesting that $InsP_3$ and caffeine- and ryanodine-sensitive Ca^{2+} stores in this tissue are separate and independent entities. This conclusion was also supported by experiments carried out with THAPS and CPA, two putative inhibitors of the SR Ca^{2+} ATPase (Seidler *et al*., 1989; Thastrup *et al*., 1990; Kirby *et al*., 1992). Pretreatment with either compound significantly reduced the amplitude of PE contractions, but had little or no effect on the amplitude of caffeine contractions. These results are similar to those of a recent study (Tribe *et al*., 1994) in cultured vascular smooth muscle cells which showed that $InsP_3$-sensitive Ca^{2+} stores were depleted by THAPS or CPA but caffeine-sensitive Ca^{2+} stores were not affected, suggesting that caffeine-sensitive Ca^{2+} stores may have a THAPS- and CPA-insensitive Ca^{2+} sequester mechanism. However, there may be considerable diversity between different types of smooth muscle with regard to the independence of these two types of SR Ca^{2+} stores, since in many cells there may be partial or complete overlap of $InsP_3$-sensitive Ca^{2+} stores and caffeine-sensitive Ca^{2+} stores (Iino *et al*., 1988; Baro & Eisner, 1992). There may also be differences between pulmonary arteries from different species and branches of the pulmonary tree. For example, Salvaterra & Goldman (1993) found that both caffeine and THAPS blocked the early release of Ca^{2+} induced by hypoxia in rat cultured pulmonary arterial cells, and De La Fuente *et al*. (1995) found that both THAPS and CPA blocked caffeine- and noradrenaline-induced contractions in rat chemically skinned main pulmonary arteries. In addition, there may even be heterogeneity within $InsP_3$-sensitive Ca^{2+} stores and caffeine-sensitive Ca^{2+} stores. The fact that nisoldipine nearly completely blocked the hypoxic contraction (Figure 6), but only reduced the caffeine contraction by some 40–45% (Figure 8), might indicate that hypoxia only stimulates Ca^{2+} release from part of the caffeine-sensitive store.

In our experiments, we obtained evidence for interactions between the two types of SR Ca^{2+} stores which indicates that the $InsP_3$-sensitive store may normally act to buffer some of the Ca^{2+} released from the caffeine- and ryanodine-sensitive Ca^{2+} store. In contrast to control tissues, in CPA-treated tissues,

there was consistently a small sustained increase in resting tension following a brief caffeine contraction in the continued presence of ryanodine. This suggests that part of the enhanced magnitude of HYP contractions observed in the presence of THAPS or CPA might be attributed to loss of a functional $InsP_3$ store which normally acts to buffer some of the Ca^{2+} released by hypoxia. Indeed, this effect may also partially explain the apparent dependence of the hypoxic contractile response in this tissue on agonist precontraction. PE-induced release of Ca^{2+} from $InsP_3$-sensitive Ca^{2+} stores would be expected to compromise the ability of this store simultaneously to take up and buffer Ca^{2+} released from the caffeine- and ryanodine-sensitive store during hypoxia (van Breemen & Saida, 1989; van Breemen *et al*., 1995).

Exposure of tissues to THAPS or CPA failed to produce any consistent change in resting tension in our experiments. In a few tissues (cf Figure 4) a small relaxation was observed which might represent some small endothelium-dependent relaxation (Fukao *et al*., 1995), since it was not observed in endothelial denuded arteries. However, even in endothelial denuded arteries no significant increase in resting tension was observed. This was somewhat surprising since these agents usually produce some small increase in tension which has been ascribed to an increase in passive Ca^{2+} efflux from intracellular stores following inhibition of the Ca^{2+}-ATPase (Thapstrup *et al*., 1990; Xuan *et al*., 1992). However, THAPS failed to produce any detectable change in resting $[Ca^{2+}]_i$ in cardiac cells (Kirby *et al*., 1992). Presumably in pulmonary artery, if these agents produce a slow enough increase in passive Ca^{2+} efflux from intracellular stores following inhibition of the Ca^{2+}-ATPase, sarcolemmal Ca^{2+} efflux mechanisms or other intracellular organelles capble of sequestering Ca^{2+}, may prevent contractile activation. The lack of any significant effect of these agents on resting tension also suggests that under our experimental conditions, these agents do not by themselves, result in activation of a capacitative Ca^{2+} entry pathway which may be activated by intracellular Ca^{2+} store depletion in some types of cells (Putney, 1990; Vaca & Kunze, 1994; Ohta *et al*., 1995).

In the presence of THAPS or CPA, HYP contractions were surprisingly potentiated. Further experiments revealed that these HYP contractions were somewhat similar to those observed under control conditions since they were partially sensitive to nisoldipine and ryanodine, but unlike control HYP contractions, a significant portion of the contractions were insensitive to these agents. Since the HYP contractions under these conditions were nearly completely blocked by removal of extracellular Ca^{2+}, this suggests that in the presence of THAPS or CPA, HYP induces Ca^{2+} entry through an additional nioldipine- and ryanodine-insensitive pathway. The nature of this novel Ca^{2+} entry pathway is not clear. Despite its apparent sensitivity to block by SK&F 96365, the effects of this compound appeared to be rather non-selective, causing significant inhibition of contractions induced by KCl, PE and caffeine. If this novel Ca^{2+} entry pathway is similar to the capacitative Ca^{2+} entry pathway which is activated by intracellular Ca^{2+} store depletion in some cells, it remains to be determined why it was not activated by THAPS or CPA alone, but was activated by hypoxia in the presence of these compounds in canine pulmonary arterial smooth muscle.

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