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Sensitivity to protein kinase C inhibitors of nicardipine-insensitive component of high K^+ contracture in rat and guinea-pig aorta

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1 In the rat and guinea-pig aorta, we observed that the contraction to hypertonically-added K^+, unlike the isotonic K^+-induced contraction, was only partially sensitive to nicardipine (0.1, 1 and 10 μM), an L-type Ca^{2+} channel blocker and occurred in Ca^{2+}-free medium containing 50 μM EGTA. We have characterized this nicarpidine-insensitive hypertonically-added K^+ contraction.

2 The contraction induced by an equi-osmolar concentration of mannitol was similar in size to that evoked by hypertonically-added K^+.

3 When the tissue was depleted of its internal Ca^{2+} stores with various agents such as phenylephrine (10 μM), cyclopiazonic acid (30 μM), thapsigargin (1 μM) or ryanodine (30 μM), or by incubation in Ca^{2+}-free medium over 30 min, little effect was observed on the high K^+ contracture in the presence of L-type Ca^{2+} channel blockade.

4 Phentolamine (10 μM) or indomethacin (10 μM) did not reduce the contraction induced by high K^+.

5 Application of a protein kinase C inhibitor, H7 (10, 30 and 100 μM) or calphostin C (1 μM), reduced the high K^+ contraction but not that caused by an equi-osmolar concentration of mannitol.

6 The data suggest that hypertonic K^+-induced contraction differs from that caused by hypertonicity or depolarization *per se* and invokes membrane enzyme activation.

Keywords: Hyperosmolarity; vascular contraction; H7; calphostin C

Introduction

It is well established that in most smooth muscle a depolarizing agent, such as high K^+, evokes contraction via an elevation of cytosolic Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels (Bolton, 1979). However, Jenkin and his coworkers (1991) demonstrated that the high K^+-induced contraction in the guinea-pig aorta was relatively insensitive to nifedipine compared to the rat aorta. Several investigators have provided evidence for the release of intracellularly-stored Ca^{2+} by high K^+ in rat aortic smooth muscle cells (e.g. Kobayashi *et al.*, 1985) and rat parotid cells (Takemura & Ohshika, 1987; 1988). In the rat vas deferens, accumulation of inositol 1,4,5-trisphosphate (IP_3) was reported to accompany the contraction induced by high K^+ and this occurred in tissue from animals treated with reserpine or in the presence of an α-adrenoceptor antagonist, prazosin (Khoyi *et al.*, 1989).

Involvement of protein kinase C (PKC) in high K^+ contraction in the rat vas deferens has also been suggested (Rice & Abraham, 1992) and this appears to be consistent with the report that high K^+ stimulates the translocation of PKC and activates it (Haller *et al.*, 1990). More recently, a study reported differential relaxation effects by Ca^{2+} channel blockers on high K^+ contracture which was induced by two commonly used techniques: iso-osmolar substitution of NaCl with KCl and hyperosmolar addition of KCl (Nielsen-Kudsk *et al.*, 1992). The findings of the present study show that hyperosmolar addition of KCl causes nicardipine-insensitive contraction in the rat and guinea-pig aorta which can be reduced by blockers of PKC.

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Methods

Male Hartley guinea-pigs (250–300 g) and Wistar rats (300–350 g) were used. The animals were killed by stunning and decapitation by use of a procedure approved by our University Animal Care Committee. The thoracic aorta was isolated and placed in Krebs solution at pH 7.4 containing (mM): NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1 and glucose 11. Fat and connective tissue were removed under a dissecting microscope and 3–4 mm rings were prepared. The endothelium was removed with the teeth of a pair of forceps and the rings were mounted on a 15 ml organ bath connected to a force transducer (Grass FT03C) and a chart recorder (Gould 2800). The presence or absence of the endothelial cells was tested functionally with 10 μM carbachol.

The organ baths and Krebs solution were bubbled continuously with 95% O_2/5% CO_2 and warmed to 37°C. The rings were equilibrated for 20 min before stretching to the optimal resting force of around 3 g for the guinea-pig aorta and 2 g for the rat aorta. Stimulation of the arteries with 120 mM K^+ added hypertonically was repeated every 15–20 min until reproducible contractions were obtained. For Ca^{2+}-free Krebs, $CaCl_2$ was replaced by 50 μM EGTA.

Cumulative concentration-response curves for K^+ were constructed in the absence or the presence of the drug of interest. Increasing concentrations of K^+ were added to the baths without omitting any other ion, i.e. producing hypertonic solutions. Substitution of 100 mM KCl for 100 mM NaCl constituted iso-osmolar solution of high K^+.

Following an equilibration period of about 1 h, all arterial rings were stimulated with increasing concentrations of K^+ to construct a concentration-response curve. The response to a maximum concentration of K^+ of this concentration-response curve was used as 100% for subsequent concentration-response curves for each arterial ring. It has been suggested that a use-dependent phenomenon is associated with repeated high K^+ stimulation in the rat aorta (Wright,

1991). Therefore, time control experiments were performed in parallel but results showed no use-dependent phenomenon.

Thapsigargin (1 μM), cyclopiazonic acid (CPA, 30 μM), ryanodine (30 μM) or calphostin C (1 μM) was added to the arterial rings and incubation continued for 60–90 min. The effects of these compounds are maximal at these concentrations (Deng & Kwan, 1991; Low *et al.*, 1991; 1992; Shimamoto *et al.*, 1993). Low Cl^- Krebs solution was made by substituting NaCl with sodium isethionate (Sigma).

Drugs

Thapsigargin (Sigma), CPA (Sigma), 12-*o*-tetradecanoylphorbol-13-acetate (TPA, Sigma), phorbol 12, 13-dibutyrate (PDBu, Sigma) and calphostin C (Kamiya Biomedical Company) were dissolved in dimethyl sulphoxide (DMSO) to make a stock solution of 10 mM. Calphostin C was protected from light and was made up fresh before use. Ryanodine (Research Biochemical Inc) and nicardipine (Sigma) were prepared in absolute ethanol as a stock solution of 10 mM. Indomethacin (Sigma) was dissolved in NaOH and the stock was titrated with HCl to pH 7.4. 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7, Sigma) was dissolved in H_2O to a stock solution of 10 mM. All other chemicals were of laboratory standard from various commercial sources. None of the solvents affected the tissue response when the pharmacological agents were omitted.

Statistical analysis

Data are expressed as the mean ± standard error of mean (s.e.mean). Significant differences were analysed by Student's paired two-tailed test or one-way analysis of variance where appropriate. When the F ratio is significant, the significantly-differing pairs were determined by Bonferroni's method. The minimal P value accepted for statistical significance was 0.05. n refers to the number of experiments from not less than three animals.

Results

Differential effects of nicardipine on high K^+-induced contraction in rat and guinea-pig aorta

Concentration-response curves to K^+ were constructed for both the guinea-pig aorta and rat aorta in parallel. In normal Krebs solution, it was observed that rat aorta had a lower threshold for contraction to K^+ elevation and reached maximum contraction at a lower K^+ concentration (Figure 1a) compared to guinea-pig aorta which was relatively less sensitive to K^+ (Figure 1b). In the guinea-pig aorta, the tissue concentration-response curve fell off at around 300 mM K^+ (not shown).

The effects of nicardipine on hypertonically-added K^+-induced contraction in the aorta of (a) rat and (b) guinea-pig are shown in Figure 1. When the tissue was pretreated with 0.1, 1 or 10 μM nicardipine prior to the construction of the concentration-response curve to K^+, the guinea-pig aorta still contracted in a concentration-dependent fashion in response to K^+. The slope of the concentration-response curve was lowered by nicardipine. Nicardipine reduced the contractile response by about 20–55% ($P<0.05$) but did not abolish the responses.

In the rat aorta, contraction to K^+ also occurred in nicardipine but it was inhibited by 40–60% ($P<0.05$) by the L-type Ca^{2+} channel antagonist. There was no significant difference between responses in the presence of varying concentrations of nicardipine.

After 5 min of pre-incubation in Ca^{2+}-free medium, increasing K^+ concentrations contracted the rat aorta: the contraction was not significantly different from that in the presence of nicardipine (Figure 1a). Similarly, the guinea-pig aorta responded in Ca^{2+}-free medium as it did in the

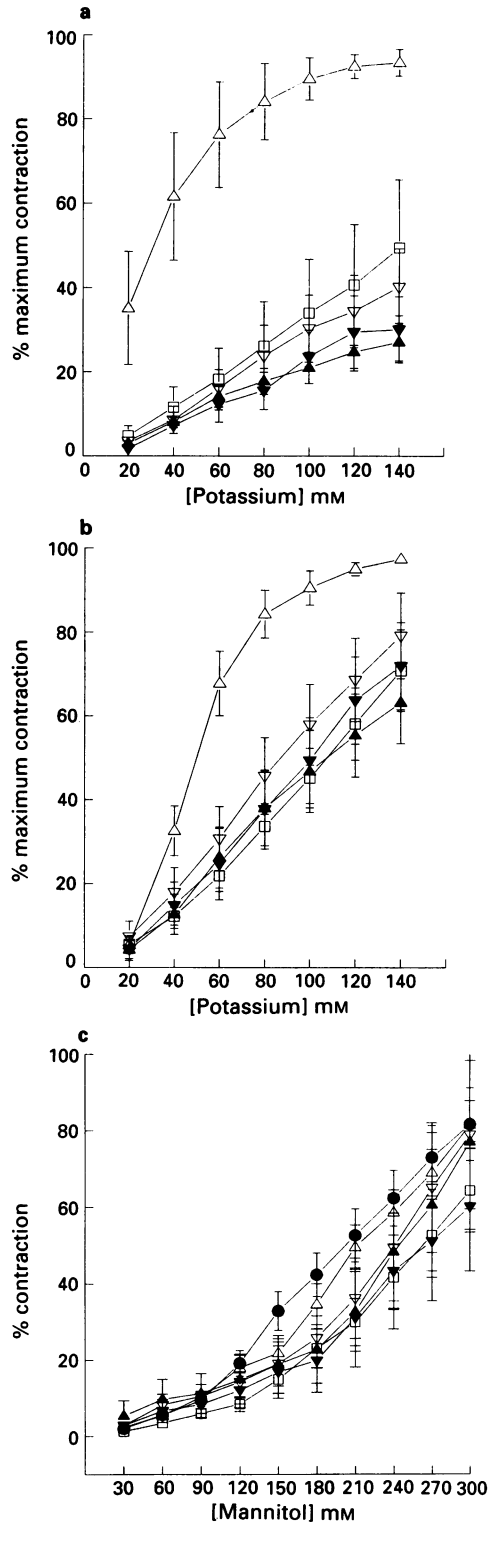

Figure 1 Concentration-dependent contraction to K^+ in the (a) rat ($n = 6$) and (b) guinea-pig aorta ($n = 8$). Some of the rat arterial rings were pre-incubated with 0.1 (▲), 1 (▽) or 10 (▼) μM nicardipine or were pre-incubated in Ca^{2+}-free Krebs solution (□) prior to the construction of a concentration-response curve to K^+. In the rat aorta, a substantially greater portion of the contraction was blocked by nicardipine or by the absence of external Ca^{2+} compared to controls (△). In contrast, a fair portion of K^+ contraction in the guinea-pig was insensitive to nicardipine or to the absence of external Ca^{2+} compared to controls (△). (c) Concentration-dependent responses to mannitol in the rat (△, $n = 18$) and guinea-pig (○, $n = 12$) aorta in normal Krebs solution. Concentration-response curves to mannitol were also constructed in the rat aorta in Ca^{2+}-free Krebs solution (□, $n = 9$) and in the presence of 0.1 (▲, $n = 6$), 1 (▽, $n = 6$) or 10 (▼, $n = 6$) μM nicardipine.

presence of nicardipine in the Ca^{2+}-containing medium (Figure 1b). In both tissues, the increase in tension with increments of K^+ concentration in Ca^{2+}-free medium was less steep compared to that in normal Krebs similar to the responses in the presence of nicardipine. After the completion of the concentration-response curve, addition of 10 mM EGTA or 3 mM nickel did not affect the contraction, confirming that the contraction did not involve an influx of Ca^{2+} from an extracellular source.

Concentration-response curves to mannitol in Ca^{2+}-containing Krebs solution, Ca^{2+}-free Krebs solution or in the presence of nicardipine (0.1, 1 or 10 μM) in the rat aorta are shown in Figure 1c. The responses to mannitol in normal Krebs solution obtained from the guinea-pig aorta are also shown in Figure 1c. The contractions are expressed as a percentage of maximum K^+ response in Ca^{2+}-containing Krebs solution. In general, while the responses to mannitol in normal Krebs solution, in Ca^{2+}-free medium and in the presence of nicardipine were overlapping, at higher concentrations of mannitol (>240 mM), contractions were significantly greater compared to those evoked by hypertonically-added K^+ under similar conditions. The size of the responses from the guinea-pig aorta were similar (not significantly different) to those evoked by hypertonically-added K^+ in Ca^{2+}-free medium or in the presence of nicardipine. Addition of 10 mM EGTA for 10 min prior to the construction of mannitol concentration-response curve in Ca^{2+}-free medium did not have a significant effect when compared to controls which were not pre-exposed to high EGTA.

Comparison between K^+ contracture induced by hypertonic addition and isotonic substitution

Figure 2 shows contraction in Ca^{2+}-free medium expressed as a percentage of contraction in the presence of 2.5 mM Ca^{2+} in rat and guinea-pig aorta. The contractions were induced by hypertonic addition of 100 mM KCl, KCl substituted isotonically for NaCl (100 mM) and KCl added hypertonically to low Cl^- Krebs solution made by isethionate substitution.

Hypertonically-added K^+ caused significantly larger contraction compared to isotonic substitution ($P<0.05$). As expected, contractions to isotonic K^+ addition in the absence of extracellular Ca^{2+} were very small. Hypertonically-added K^+ to normal Krebs doubles the Cl^- ion concentration. So, to investigate if this ion contributes to the 'hypertonic effect', NaCl was substituted with sodium isethionate. In this modified Krebs solution, referred to as 'low Cl^- solution', the only ion that was elevated was the K^+ ions when KCl was added hypertonically. Under these conditions, the contraction was significantly potentiated compared to results obtained when KCl was added hypertonically ($P<0.05$). Therefore, the contraction induced by hypertonically-added KCl in normal Krebs medium was not due to an increased concentration of Cl^- ions.

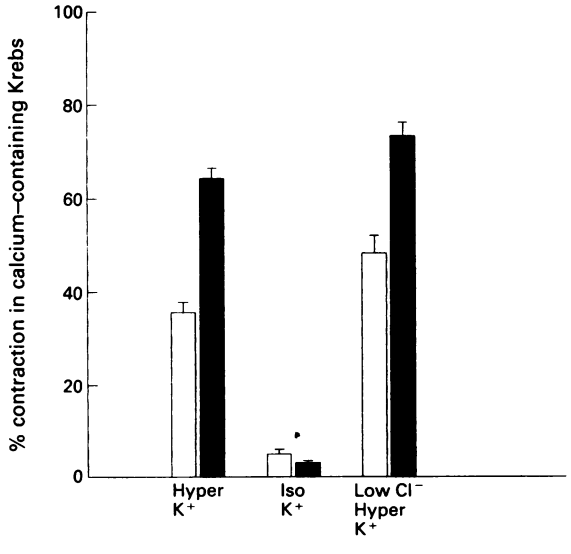

Figure 2 Aortic rings of rat (open columns, $n = 13$) and guinea-pig (solid columns, $n = 17-23$) aorta were stimulated in Ca^{2+}-free Krebs with high K^+ (100 mM) either added hypertonically (Hyper K^+) or isotonically-substituted (Iso K^+). Responses are expressed as a percentage of the contraction evoked when 2.5 mM Ca^{2+} was added to the bath. 'LowCl Hyper K^+' denotes hypertonically-added K^+ to low Cl^- Krebs medium. Contraction by LowCl Hyper K^+ was significantly larger than Hyper K^+ alone. Iso K^+ is significantly less compared to all groups.

Lack of effects of pretreatment with phentolamine and indomethacin

Inclusion of 10 μM phentolamine, an α_1- and α_2-adrenoceptor antagonist, did not affect significantly the K^+-induced contraction eliminating the possibility of noradrenaline release from depolarized nerve terminals observed to be present on the guinea-pig aorta (Morris, 1991). Furthermore, since the contraction persisted in Ca^{2+}-free medium, it is unlikely that any release of neurotransmitter occurred.

Indomethacin was used to inhibit phospholipase A_2, the enzyme responsible for production of such potent vasoconstrictor prostanoids as thromboxane A_2. After an incubation period of 30 min with 10 μM indomethacin, there was no significant change on the concentration-responses to elevated K^+ in either normal or Ca^{2+}-free Krebs solution in either rat or guinea-pig aorta (not shown).

Role of intracellular Ca^{2+} stores

Various modulators of the internal Ca^{2+} stores were used to investigate the possible contribution of the internal Ca^{2+} stores to hypertonically-added K^+-induced contraction. Guinea-pig aortic rings were treated with CPA (30 μM), thapsigargin (1 μM) or ryanodine (30 μM). CPA and ryanodine had no significant effect on K^+ concentration-response curves in normal Krebs solution compared to controls (Figure 3a). On the other hand, pretreatment with thapsigargin potentiated the responses to high K^+ significantly compared to controls ($P<0.05$).

Although CPA, thapsigargin or ryanodine apparently shifted significantly the concentration-response curve to K^+ in the presence of nicardipine (10 μM) when compared with untreated controls in the presence of nicardipine (Figure 3b), the responses of treated tissue in the presence and absence of nicardipine were not significantly different (cf. CPA, thapsigargin and ryanodine in Figure 3a and Figure 3b). Therefore, this may indicate that nicardipine had no effect on the concentration-response curves to high K^+ in the presence of CPA, thapsigargin or ryanodine. However, nicardipine inhibited significantly the contraction to hypertonically-added K^+ by about 20–40% (cf. control in Figure 3a and Figure 3b) as was the case in Figure 1b. It is also interesting to note the lack of inhibition by nicardipine in CPA, thapsigargin or ryanodine-treated tissues on concentration-response curves to K^+, suggesting that these compounds can induce Ca^{2+} influx through non-L-type Ca^{2+} channels, thereby enhancing contraction due to elevated K^+. These observations are consistent with results which have previously been reported that modulators of the Ca^{2+} release and uptake mechanisms of the SR can induce Ca^{2+} influx through non-dihydropyridine-sensitive channels (Low *et al.*, 1991; Xuan *et al.*, 1992).

In another series of experiments, the tissues were incubated in Ca^{2+}-free medium for 5 min prior to a challenge with 10 μM phenylephrine (Figure 3c). In controls, the transient contraction was followed by a contraction which was lower in amplitude and longer in duration and the latter was manifest by a 25% increase in basal contraction as seen in

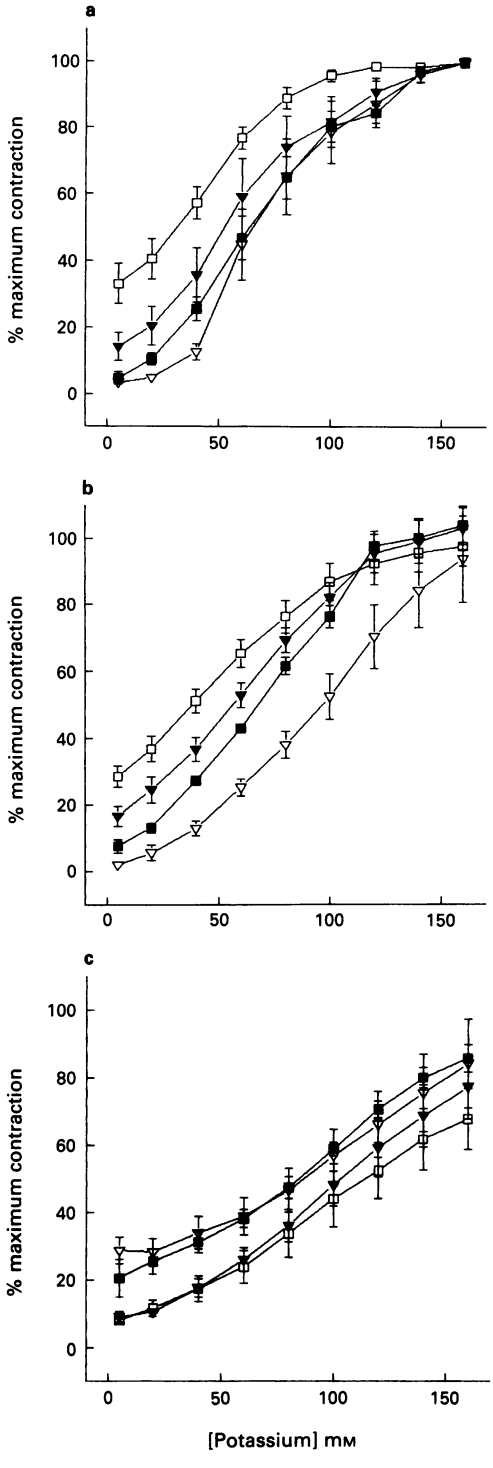

Figure 3 Concentration-dependent responses to K^+ in the guinea-pig aorta in the absence (∇, $n = 5$) and presence of the modulators of internal Ca^{2+} uptake (cyclopiazonic acid (CPA), 10 μM, ▼, $n = 5$; thapsigargin, 1 μM, □, $n = 6$) and release (ryanodine, 30 μM, ■, $n = 5$). (a) In normal Krebs solution, there was no significant effect of CPA or ryanodine on the concentration-dependent responses to K^+. Thapsigargin significantly enhanced K^+ contraction. (b) Procedure is as in (a) except, nicardipine (10 μM) was present. The responses obtained from pretreatment with CPA, ryanodine or thapsigargin were similar to those in (a). The control responses to K^+ were inhibited by nicardipine. Therefore, the apparent enhancement of responses by CPA, ryanodine or thapsigargin could be accounted for by the effect of nicardipine on the control. (c) Procedure as in (a) except the experiments were carried out in Ca^{2+}-free Krebs after the stimulation of the tissue with phenylephrine (10 μM). Phenylephrine induced a transient contraction followed by a fairly sustained contraction in Ca^{2+}-free medium in control. In the rings treated with CPA or thapsigargin, phenylephrine-induced contraction (both transient and sustained) was absent. In the ryanodine-treated tissue, the transient was absent, although the sustained contraction due to phenylephrine was not blocked. None of these manipulations affected the nicardipine-insensitive high K^+ contraction.

Figure 3c at 5 mM K^+ (significantly different from responses of CPA- or thapsigargin-treated tissues). The transient contraction associated with the addition of phenylephrine was absent in the tissue-pretreated with CPA, thapsigargin or ryanodine but present in controls. While the transient contraction was absent in ryanodine-treated tissues, phenylephrine stimulation in these tissues gave rise to a sustained contraction of lower amplitude which was not significantly different from control responses. The maximum amplitude of hypertonically-added K^+ contraction in Ca^{2+}-free medium was not significantly different in tissues pretreated with CPA, thapsigargin or ryanodine compared with controls. These results suggest that the ryanodine-sensitive Ca^{2+} pool may be different from that which is sensitive to CPA, thapsigargin and phenylephrine in the guinea-pig aorta.

Phenylephrine still induced a transient contraction when the tissue was incubated for around 5 min in Ca^{2+}-free medium. In order to ensure that the internal Ca^{2+} stores were emptied particularly when there was some evidence for multiple Ca^{2+} stores in this tissue (guinea-pig aorta), pre-incubation of the tissue in Ca^{2+}-free medium containing 50 μM EGTA was prolonged from 5 to 30 min. Successful depletion was denoted by the absence of phenylephrine-induced contraction. The concentration-response curves to K^+ constructed after 5 or 30 min were not significantly different (not shown). These observations together with those made with the selective modulators of the internal Ca^{2+} stores eliminate any direct participation of intracellular stores in sustaining hypertonically-induced K^+ contraction in Ca^{2+}-free medium.

Effects of H7

Contractions in the rat and guinea-pig aorta were induced by hypertonically added 100 mM K^+ or equi-osmolar concentration of mannitol in Ca^{2+}-free Krebs solution. This contraction was expressed as 100%. Figure 4 shows that in the guinea-pig and rat aorta, H7, a non-selective inhibitor of protein kinases, relaxed in a concentration-dependent manner the arterial rings contracted with hypertonically-added K^+. It had little effect on the contraction to hypertonic mannitol. Addition of H7 on the plateau of mannitol-induced contraction caused a further increase in tension in the guinea-pig aorta. Larger maximum relaxations (~65%) to H7 were observed in the rat aorta compared to those observed in the guinea-pig aorta (~12%). The relaxation in the rat but not in the guinea-pig aorta was statistically significant.

Effects of calphostin C

The participation of PKC in the high K^+-induced contraction was further investigated with a highly selective inhibitor of PKC, calphostin C (Shimamoto *et al.*, 1993) in the guinea-pig aorta. Figure 5 shows the results obtained with calphostin C. Pre-incubation with calphostin C (1 μM) significantly inhibited the maximum high K^+ contraction in normal Krebs solution by about 25% ($P<0.05$, Figure 5a) and by about 50% in Ca^{2+}-free solution ($P<0.05$, Figure 5b). In both cases, inhibition by calphostin C occurred at KCl concentrations greater than 60 mM. Following stimulation of the arterial rings with phenylephrine (10 μM), hypertonic addition of KCl caused concentration-dependent increase in contraction. In the presence of calphostin C, the phenylephrine and KCl-induced responses at all concentrations tested were reduced by about 20% ($P<0.05$, Figure 5c).

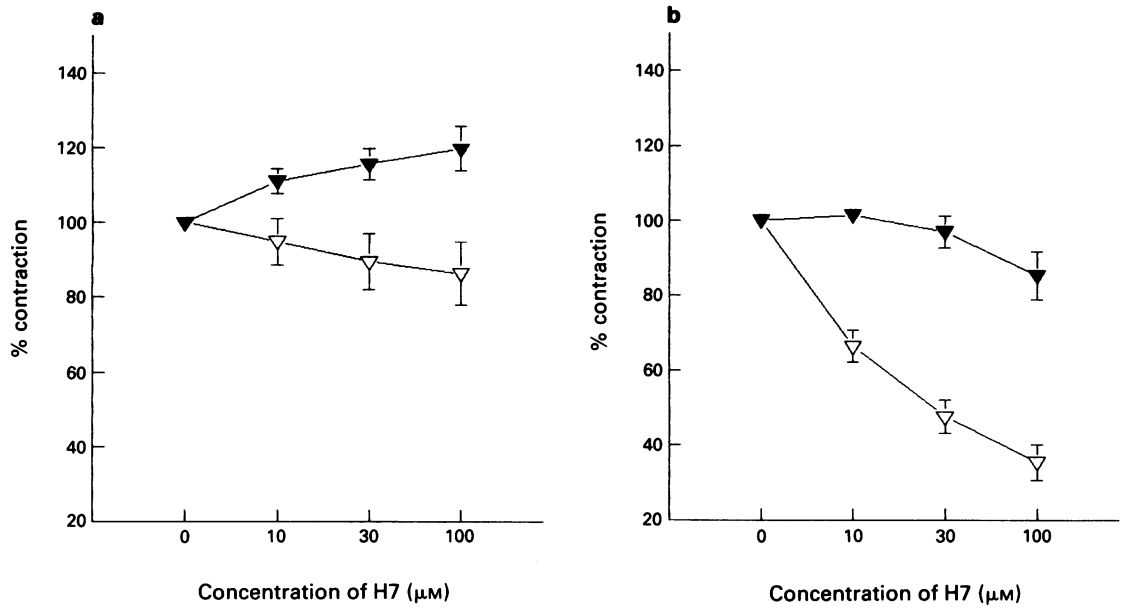

Figure 4 Concentration-dependent relaxation effect of H7 in (a) guinea-pig ($n = 9-10$) and (b) rat ($n = 11-12$) aorta to hypertonically-added K^+ (100 mM, ▽) or mannitol (200 mM, ▼). Contraction to hypertonically-added K^+ or mannitol in Ca^{2+}-free Krebs solution is represented as 100%.

Parallel studies using TPA (1 μM) and PDBu (1 μM), activators of PKC were performed. In guinea-pig aortic rings pretreated with calphostin C (1 μM), PDBu-induced contractions were significantly inhibited (14.7 ± 5.3% of maximum K^+ contraction in Ca^{2+}-containing Krebs solution, $n = 6$) compared to controls (54.3 ± 8.9%, $n = 6$). In two guinea-pig aortic rings, TPA-induced contractions were completely inhibited while control rings underwent a mean contraction of 35.7 ± 9.9% ($n = 2$).

It has been reported that PKC activation can either be dependent or independent of external Ca^{2+} and Ca^{2+} release. Therefore, in order to remove Ca^{2+} completely, the arterial rings were pre-incubated with high EGTA (10 mM) for at least 10 min prior to the construction of concentration-response curve to high K^+. These tissues could no longer respond to phenylephrine in Ca^{2+}-free Krebs solution. Contraction to hypertonic KCl still persisted under these conditions (data not shown). This suggests that activation of PKC in the guinea-pig aorta may well be a Ca^{2+}-independent mechanism.

Discussion

Hypertonically-added KCl causes vascular contraction independent of Ca^{2+} and insensitive to nicardipine

Influx of Ca^{2+} through the voltage-dependent, L-type Ca^{2+} channels occurs following depolarization in smooth muscle. KCl is a depolarizing agent commonly used by hypertonic addition, but it can also be applied by isotonic substitution. In this study, we have shown that in the rat and guinea-pig aorta, contractions produced by these two techniques occurred via different mechanisms. This finding is consistent with results reported by Nielsen-Kudsk *et al.* (1992) that contraction induced by isotonic substitution of KCl could be wholly antagonized by Ca^{2+} channel blockers, but contraction induced by hypertonic addition could only be partially antagonized. The nicardipine-sensitive component of high K^+ contraction accounts for approximately 50% and 20%, respectively, of the contractions of rat and guinea-pig aorta in normal Krebs solution containing 2.5 mM Ca^{2+}.

Nicardipine-insensitive KCl contraction cannot be solely explained by hyperosmolarity

Nielsen-Kudsk *et al.* (1992) suggested that hyperosmolarity *per se* contributes to contraction when KCl was added hypertonically in guinea-pig airway smooth muscle. In the present study, although the contraction induced by mannitol of equal osmolarity to that of KCl was similar to or larger than that induced by hypertonically-added K^+, the contraction induced by mannitol was resistant to relaxation by H7. This shows that the mannitol-induced contraction does not depend on an H7-sensitive kinase. It also suggests that contracture induced by hypertonically-added K^+ in vascular smooth muscle cannot be wholly accounted for by hyperosmolarity *per se*. Instead, this contracture appears to be mediated by more than one mechanism.

Nicardipine-insensitive KCl contraction is sensitive to PKC inhibitors

PKC is present at high concentrations in smooth muscle and can be activated by diacylglycerol or phorbol esters at near resting intracellular Ca^{2+} levels and remains active in the absence of Ca^{2+} (Nishizuka, 1984; 1992). It is a cytosolic protein the activation of which has been directly demonstrated to involve its low Ca^{2+}-dependent movement from the cytosol to the surface membrane (Khalil & Morgan, 1991). A fraction of the basal tone of vascular smooth muscle has been suggested to involve the activation of Ca^{2+}-independent isozyme of PKC (Collin *et al.*, 1992). This observation is consistent with a report on various arterial tissues including the rat aorta, carotid artery, tail artery, rabbit aorta and mesenteric artery, that a phorbol ester, 12-deoxyphorbol 13-isobutyrate, caused contraction independent of a rise in intracellular Ca^{2+} and this was thought to be due to increased cross-bridge cycling, independent of the phosphorylation of the myosin light chain (Sato *et al.*, 1992).

In this study, we provide evidence for a role played by PKC in hypertonically-induced KCl contraction. Hypertonically-added K^+ appears to cause contraction in part via the activation of PKC since the contraction was blocked by the PKC inhibitors, H7 and calphostin C. These compounds inhibit PKC activation by interacting with the

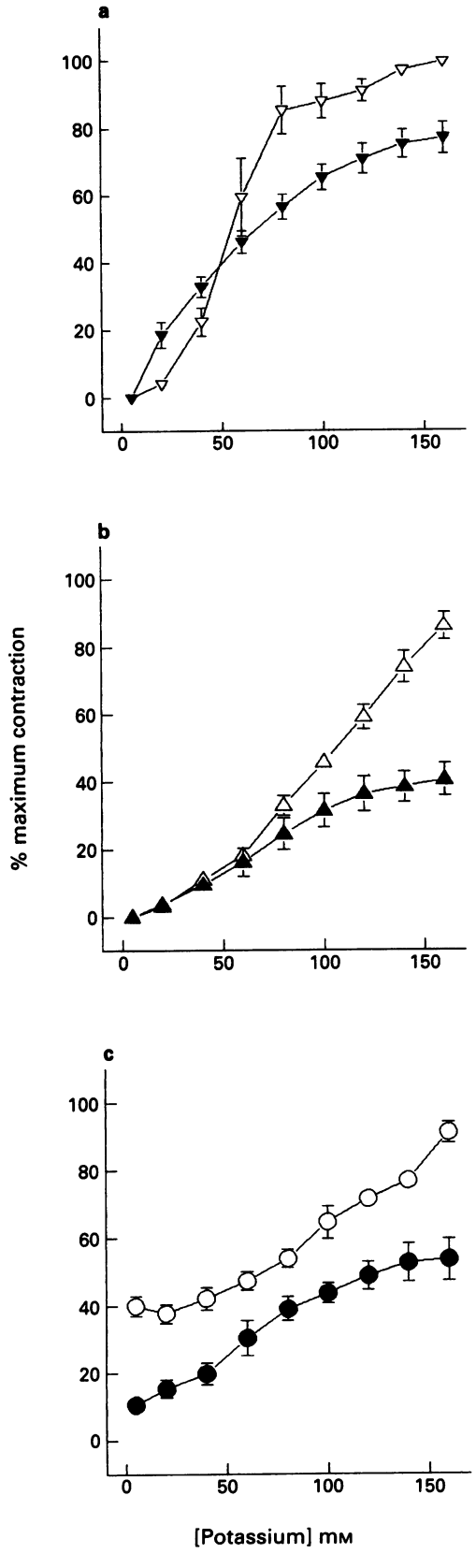

Figure 5 Effects of calphostin C (1 μM) on the concentration-responses to K^+ in the guinea-pig aorta. (a) In normal Krebs solution ($n = 6$), calphostin C (▼) reduced the responsiveness of the tissue by about 20% compared to controls (▽). (b) In Ca^{2+}-free medium ($n = 6$), contraction to high K^+ was significantly inhibited by 50% in calphostin C (▲)-treated tissues compared with controls (△). (c) Pretreatment of the tissue with calphostin C (●) significantly reduced the phenylephrine- and KCl-induced contractions in Ca^{2+}-free medium ($n = 6$) compared with controls (○).

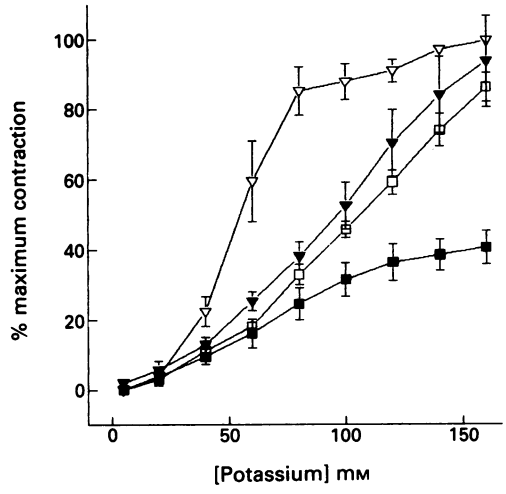

Figure 6 A summary of the main findings of this study in the guinea-pig aorta: components of hypertonically-added K^+ contraction. The relationship of concentration-responses to K^+ in normal Krebs solution ($n = 8$, ▽), in Krebs containing nicardipine (10 μM, $n = 8$, ▼), in Ca^{2+}-free Krebs ($n = 6$, □) and in Ca^{2+}-free Krebs containing calphostin C (1 μM, $n = 6$, ■).

catalytic (Hidaka *et al.*, 1984) or regulatory diacylglycerol binding domain, respectively (Kobayashi *et al.*, 1989).

Nicardipine-insensitive KCl contraction is not due to Ca^{2+} release from the intracellular store

A recent report demonstrated that a large membrane hyperpolarization can inhibit noradrenaline-induced synthesis of inositol 1,4,5-trisphosphate (IP_3) in the rabbit mesenteric artery although depolarization did not appear to affect the synthesis of IP_3 in the study (Itoh *et al.*, 1992). On the other hand, the release of internally-stored Ca^{2+} by high K^+ in other smooth muscle has been demonstrated directly with a fluorescent dye in a Ca^{2+}-free environment (rat aorta; Kobayashi *et al.*, 1985) or indirectly by measurement of IP_3 levels after ruling out effects from neurotransmitter release (rat vas deferens; Khoyi *et al.*, 1989). In non-excitable cells such as the rat parotid cells, high K^+ elevated free Ca^{2+} concentration in both normal and Ca^{2+}-free medium. It was concluded that this results from the mobilization of Ca^{2+} from internal storage sites since high K^+ did not affect $^{45}Ca^{2+}$ uptake into the cells (Takemura & Ohshika, 1987; 1988). Their findings suggest that high K^+ can activate phospholipase C in some vascular muscles. The mechanism by which a depolarizing agent, such as K^+, activates membrane-bound or intracellular enzymes is not known but such a phenomenon has been previously reported (Itoh *et al.*, 1992; Quast, 1993). This interesting phenomenon in excitation-contraction coupling warrants further investigation.

In our experiments, using agents which affect uptake and release from Ca^{2+} stores, we were not able to attribute the high K^+-induced contraction in Ca^{2+}-free medium directly to any form of Ca^{2+} release from storage sites. This is expected since the release of Ca^{2+} is transient and limited by the available amount of stored Ca^{2+}. Nevertheless, the transient release of Ca^{2+} can activate PKC and phosphorylate the myosin light chain kinase. These events may possibly contribute to the sustained contraction but are unlikely to play a role in this study. When we performed an experiment in which the arteries were incubated with 10 mM EGTA to remove all the intracellular Ca^{2+} stores prior to the hypertonic addition of K^+, a concentration-dependent increase in tension was still observed. Furthermore, in most of the experiments, by the time we added H7 or calphostin C, at least 30 min had lapsed with the arterial rings bathed in

Ca^{2+}-free Krebs. A 30 min period of incubation in Ca^{2+}-free medium containing 50 μM EGTA was found to abolish the phenylephrine-induced contraction, an observation which suggests that most of the Ca^{2+} in the cell had dissipated.

Collins and her co-workers (1992) have suggested the presence of Ca^{2+}-independent isozyme of PKC in ferret aortic cells. Such an isozyme could account for our observations on high K^+ induced contraction. Since the activation of phospholipase C not only gives rise to IP_3 but also to diacylglycerol, it is not surprising that high K^+-induced depolarization can also activate PKC. It is interesting that in the guinea-pig aorta, calphostin C also significantly inhibited phenylephrine induced contraction in Ca^{2+}-free medium (Figure 5).

Summary

Figure 6 summarizes the components of contraction induced by hypertonically-added K^+ in the guinea-pig aorta. The EC_{50} of the concentration-response curve to hypertonically-added K^+ is around 50 mM in normal Krebs solution whereas in Ca^{2+}-free Krebs or with nicardipine pretreatment, the EC_{50} is around 100 mM. The addition of calphostin C at 1 μM reduced the maximum contraction by about 50%. The nature of the residual contraction in Figure 6 following blockade by calphostin C was not investigated in this study but may be accounted for by hyperosmolarity *per se*.

Results from the present study also suggest that the ryanodine-sensitive Ca^{2+} pool may be different from that which is sensitive to CPA, thapsigargin and phenylephrine in the guinea-pig aorta. Therefore, multiple Ca^{2+} stores may exist in this tissue just as in dog mesenteric artery (Low *et al.*, 1992) and other tissues (bovine adrenal chromaffin cells, Robinson & Burgoyne, 1991; sea urchin egg, Rakow & Shen, 1990 and guinea-pig papillary muscle, Lynch III, 1991).

Conclusion

Our results suggest that hypertonically-added K^+, in contrast to isotonically-added K^+, causes contraction not only via L-type Ca^{2+} channel but also by activating membrane-bound enzymes. This mechanism is not the same as that by which hyperosmolar mannitol causes contraction. A significant portion of the high K^+ contracture in Ca^{2+}-free medium can apparently be accounted for by an isozyme of PKC independently of extracellular or intracellular Ca^{2+}.

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