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Activation by high potassium of a novel voltage-operated Ca^{2+} channel in rat spleen

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1 High potassium produced a concentration-dependent contraction in rat isolated spleen.

2 The high potassium-induced contraction of rat spleen was abolished in Ca^{2+}-free Krebs solution containing 1 mM EGTA, and the subsequent addition of 3 mM Ca^{2+} restored the high potassium-induced contraction to the control level.

3 Nifedipine, verapamil, diltiazem, Cd^{2+}, Ni^{2+}, Co^{2+}, **R**-(+)-Bay K 8644 and pimozide inhibited and relaxed high potassium-induced contraction of rat spleen with IC_{50} and EC_{50} values much higher than those values in rat aorta.

4 In addition, high potassium-stimulated contraction of rat spleen was insensitive to ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-agatoxin IVA.

5 The high potassium-induced contraction of rat spleen was also unaffected by tetrodotoxin (TTX), prazosin, chloroethylclonidine (CEC), yohimbine, propranolol, atropine, diphenhydramine, cimetidine, ketanserin, 3-tropanyl-indole-3-carboxylate, saralasin, indomethacin, nordihydroguaiaretic acid, GR32191B, domperidone, naloxone, chlorpromazine, suramin, (±)-2-amino-5-phosphonopentanoic acid, 6,7-dinitroquinoxaline-2,3-dione (DNQX), L-659,877, L-703,606, lorglumide, PD 135,158 N-methyl-D-glucamine, benextramine, amiloride, dantrolene, TMB-8, econazole, staurosporine and neomycin.

6 Forskolin and sodium nitroprusside relaxed high potassium-induced contraction of rat spleen with EC_{50} values of 0.55 ± 0.04 and 20.0 ± 2.7 μM, respectively.

7 It is concluded that high potassium may activate a novel, pharmacologically uncharacterized voltage-operated Ca^{2+} channel in rat spleen.

Keywords: High potassium; voltage-operated Ca^{2+} channel; rat spleen

Introduction

The rise in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is the principal mechanism that initiates contraction in smooth muscles. Ca^{2+} binds to calmodulin, and the association of Ca^{2+}-calmodulin complex with myosin light-chain kinase activates this enzyme resulting in the phosphorylation of myosin and the concomitant activation of the myosin Mg^{2+}-ATPase by actin and the muscle to contract (Allen & Walsh, 1994; Somlyo & Somlyo, 1994). In contrast, a fall in $[Ca^{2+}]_i$ inactivates myosin light-chain kinase and permits dephosphorylation of myosin light chain by myosin light-chain phosphatase, thus deactivating the actomyosin ATPase and causing relaxation (Allen & Walsh, 1994; Somlyo & Somlyo, 1994).

The major pathway for entry of Ca^{2+} into smooth muscle is implemented by voltage-operated Ca^{2+} channels (VOC). The resting membrane potential of smooth muscle is negative (−40 to −70 mV) with respect to extracellular space. Depolarization can open VOC, causing Ca^{2+} influx to increase $[Ca^{2+}]_i$ and trigger contraction (Somlyo & Himpens, 1989). Based on pharmacological, electrophysiological and molecular cloning studies, multiple classes of VOC, which have different distributions, voltage-dependence, kinetics of activation/inactivation and conductance, have thus far been defined. By use of specific channel blockers, e.g., 1,4-dihydropyridines, ω-agatoxin IVA, ω-conotoxin GVIA, ω-conotoxin MVIIC and low concentrations of Ni^{2+} and Cd^{2+}, the high voltage-activated Ca^{2+} channels have been subdivided into L-, N-, O-, P-, Q- and R-types (Zhang *et al*., 1993; Olivera *et al*., 1994). In a preliminary study, we found that high potassium stimulated contrations of rat spleen in a concentration- and extracellular Ca^{2+}-dependent manner. In the present study, we obtained the pharmacological profile of this contraction and proved that high potassium might activate a novel, pharmacologically unidentified VOC in rat spleen.

Methods

Tissue preparation

Male Wistar rats, 250–300 g, were humanely killed. The thoracic aorta and spleen were isolated and excess fat and connective tissue were removed. The vessels were cut into rings of about 5 mm in length and endothelium was removed by rubbing with a cotton ball; the absence of acetylcholine-induced relaxation was taken as an indicator that vessels were denuded successfully. The spleens were bisected transversely into two portions. Tissues were attached to a holder under a resting tension of 1 g and equilibrated in Krebs solution for 90 min with three changes of Krebs solution before experiments were started.

Solutions

The Krebs solution contained (mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $CaCl_2$ 1.9 and $NaHCO_3$ 25.0. High potassium solutions were made either by substituting the NaCl in the normal solution with equimolar potassium (isosmotic solution), or by adding potassium to the normal solution directly (hyperosmotic solution). The solutions were aerated with a 95% O_2-5% CO_2 mixture.

Muscle tension

Muscle tension was recorded isometrically with a force-displacement transducer connected to a Grass polygraph. High

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potassium-induced contractions were performed by adding potassium to Krebs solution directly (hyperosmotic) or by exchanging the Krebs solution with isosmotic high potassium solution. The contractile effects of Ca^{2+} were studied in tissues stabilized in 80 mM isosmotic high potassium solution without Ca^{2+}. Ca^{2+} was then added from stock dilutions to obtain the desired concentrations, and the effect of each Ca^{2+} concentration was recorded. The maximal tension attained at 3 mM Ca^{2+} was considered as 100%. Usually, the inhibitors were added 15 min before the application of potassium, or cumulatively applied when the contractile tension induced by potassium reached a steady level. The concentration of inhibitors required to induce 50% inhibition (IC_{50}) or 50% relaxation (EC_{50}) of high potassium-stimulated contraction were calculated from concentration-inhibition and concentration-relaxation curves of each inhibitor, respectively. The linear portion of such curves (often approximately linear between 20% and 80% of the maximum response) were subjected to linear regression. The IC_{50} and EC_{50} values were determined by interpolation from the regression lines.

Statistics

Results of the experiments are expressed as the mean ± s.e.mean and accompanied by the number of observations. Statistical significance was assessed by unpaired Student's *t* test and *P* values less than 0.05 were considered significant.

Drugs

The following drugs were used: KCl, diltiazem HCl, neomycin sulphate, econazole HCl, tetrodotoxin (TTX), nordihydroguaiaretic acid, yohimbine HCl, prazosin HCl, indomethacin, chlorpromazine HCl, naloxone HCl, saralasin acetate, nickel chloride (Ni^{2+}), cobalt chloride (Co^{2+}) and cadminum chloride (Cd^{2+}) were obtained from Sigma Chemical Co.; chloroethylclonidine diHCl (CEC), (±)-2-amino-5-phosphonopentanoic acid, 6,7-dinitroquinoxaline-2,-3-dione (DNQX), 3-tropanyl-indole-3-carboxylate HCl, domperidone, suramin hexasodium, ketanserin tartrate, [1S-[1α,2β[S*(S*)],4α]]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[[1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]-propyl]amino]-1-phenylethyl]amino]-4-oxo-butanoic acid N-methyl-D-glucamin salt (PD135,158) 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-trifluoromethyl)-phenyl]-3-pyridine carboxylic acid (**R**-(+)-Bay K 8644), cyclo(Gln-Trp-Phe-Gly-Leu-Met) (L-659,877), *cis*-2-(diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate (L-703,606), staurosporine, amiloride HCl, dantrolene sodium, lorglumide sodium, benextramine and pimozide were from Research Biochemicals International; ω-agatoxin IVA, ω-conotoxin GVIA and ω-conotoxin MVIIC were purchased from Peptide Institute.

Results

High potassium-induced contractions in isosmotic and hyperosmotic solutions

The contractile effect of potassium on rat spleen was firstly evaluated in isosmotic (NaCl was replaced by KCl in an equimolar amount) and hyperosmotic (KCl added to normal Krebs solution directly) solution. The potency (EC_{50} values) and efficacy (maximal contractile force) of potassium-induced contractions of rat spleen did not differ significantly between solutions. The EC_{50} values and maximal contractile responses of potassium-induced contractions of rat spleen in isosmotic and hyperosmotic solutions were 46.5±3.5 ($n=6$) and 46.4±4.1 mM ($n=276$), and 0.28±0.03 ($n=6$) and 0.21±0.01 g ($n=276$), respectively (Figure 1). High potassium also caused contraction of rat aorta denuded of endothelium. The EC_{50} values and maximal contractile forces were 21.0±0.9 mM and 2.12±0.18 g ($n=6$), respectively in isosmotic solution, and 26.1±3.4 mM and 2.14±0.07 g ($n=276$), respectively in hyperosmotic solution (Figure 1). Thus, direct application of potassium into Krebs solution to induce contractions of smooth muscles was used in the further studies.

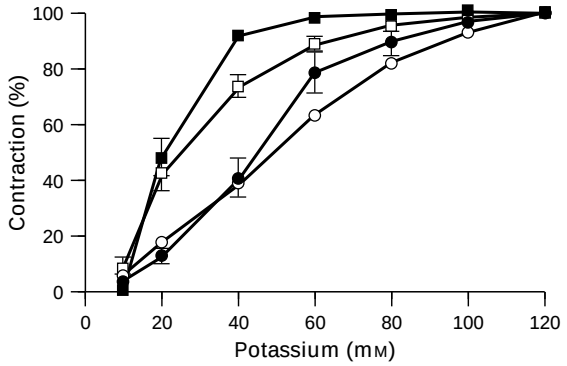

Figure 1 Concentration-response curves of high potassium-induced contractions of rat spleen and aorta in hyperosmotic and isosmotic solutions. After equilibration of spleen (circles) or aorta (squares) in Krebs solution for 90 min, various concentrations of potassium (open symbols) or isosmotic high potassium Krebs solutions (solid symbols) were used to trigger the contraction. The magnitude of the maximal contraction was taken as 100%. Each point represents the mean and vertical lines show s.e.mean ($n=6$ or 276).

Effect of extracellular Ca^{2+} on high potassium-induced contractions of rat spleen and aorta

As shown in Figure 2a, pretreatment of the rat spleen and aorta with Ca^{2+}-free Krebs solution containing 1 mM EGTA for 15 min almost completely abolished the high potassium (10–120 mM)-induced contractions. However, addition of 3 mM Ca^{2+} restored the high potassium-induced contraction to the control level.

In Ca^{2+}-free Krebs solution containing high potassium (80 mM), the cumulative addition of Ca^{2+} (0.03–3 mM) caused a stepwise increase in contraction in rat spleen and aorta. The EC_{50} values were calculated to be 0.86±0.16 and 0.54±0.05 mM, respectively ($n=6$, Figure 2b).

Effects of Ca^{2+} channel blockers on high potassium-induced contraction

L-type Ca^{2+} channel antagonists, nifedipine (0.003–0.03 μM), verapamil (0.01–1 μM), diltiazem (0.01–3 μM) and **R**-(+)-Bay K 8644 (0.1–1 μM) inhibited high potassium-induced contraction of rat aorta in a concentration-dependent manner. The IC_{50} values were calculated to be 0.014±0.003, 0.14±0.02, 0.47±0.03 and 0.48±0.09 μM at a potassium concentration of 80 mM, respectively (Figure 3a for nifedipine and Table 1). In contrast, nifedipine at a concentration of 1 μM inhibited high potassium (80 mM)-induced contraction of rat spleen by 40±4% (Figure 3b). Increasing concentration of nifedipine to 10 μM did not cause more pronounced inhibition on potassium-induced contraction in rat spleen. Thus, the IC_{50} ratio of nifedipine on the inhibition of high potassium-induced contractions of rat spleen and aorta was more than 714. Moreover, verapamil, diltiazem and **R**-(+)-Bay K 8644 also inhibited high potassium (80 mM)-induced contraction in a concentration-dependent manner with IC_{50} values 40.6±3.0, 35.7±8.0 and 27.4±1.4 μM that were 294, 77 and 58 fold higher, respectively, than their IC_{50} values in rat aorta (Table 1).

The inorganic Ca^{2+} channel blockers, Cd^{2+}, Ni^{2+} and Co^{2+} inhibited high potassium-stimulated contractions of rat spleen

and aorta in a concentration-dependent manner. Again, the IC_{50} values of Cd^{2+} and Ni^{2+} for inhibition of potassium-induced contraction of rat spleen were 38 and 6.3 fold higher than in rat aorta (Table 1). In contrast, Co^{2+} at a concentration of 1 mM inhibited 31% of high potassium (80 mM)-induced contraction in the spleen and increasing its concentration to 10 mM did not cause more pronounced inhibition on the contraction. The inhibitory effect of T- and L-type Ca^{2+} channel antagonist, pimozide was also investigated. A 2.6 times higher concentration of pimozide was necessary to inhibit the high potassium-induced contraction in spleen than in aorta (Table 1).

In rat spleen and thoracic aorta, potassium (80 mM) caused a sustained contraction lasting for at least 60 min. Cumulative application of Ca^{2+} channel antagonists at the time contractile tension reached a steady state (5 min after exposure to potassium), led to a concentration-dependent relaxation (Figure 4a for nifedipine). The concentrations of the Ca^{2+} channel antagonists that caused 50% relaxation (EC_{50} value) of the potassium-induced contraction were calculated and are listed in Table 2. Again, much higher concentrations of Ca^{2+} channel antagonists were needed to relax the high potassium-stimulated contraction in the spleen than in the aorta. However, the maximal relaxations caused by nifedipine (30 μM), Ni^{2+} (10 mM) and Co^{2+} (10 mM) were less than 20% in rat spleen.

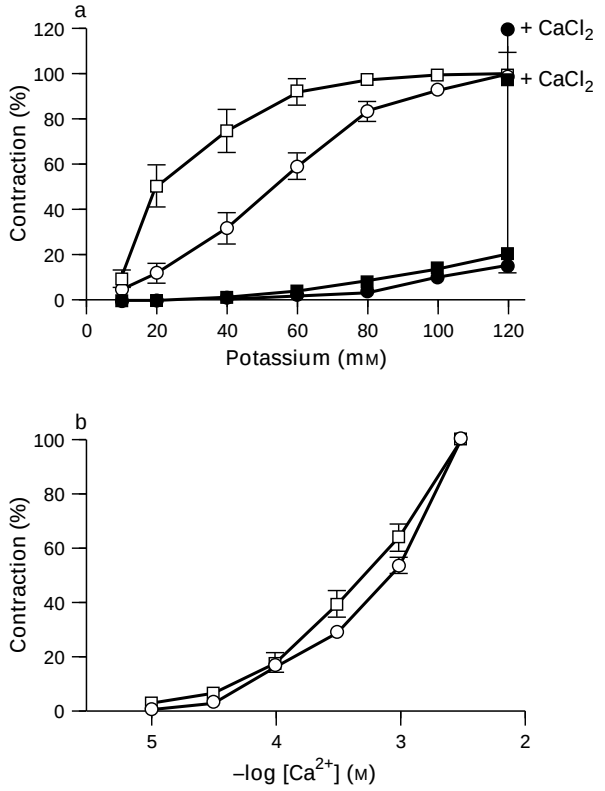

Figure 2 (a) Effects of a 15-min preincubation in Ca^{2+}-free Krebs solution (containing 1 mM EGTA) on the concentration-response curve of high potassium in rat spleen and aorta. Control tissues (Ca^{2+} 1.9 mM; spleen ○, aorta □); Ca^{2+}-free EGTA-treated tissues (spleen ●, aorta ■). The vertical line on the right side of the figure represents the contraction observed when 3 mM Ca^{2+} was replaced into the high-potassium, Ca^{2+}-free solution. (b) Effects of Ca^{2+} on high potassium-induced contractions in rat spleen and aorta. Spleen (○) or aorta (□) was preincubated in Ca^{2+}-free Krebs solution containing 80 mM potassium for 15 min, then cumulative concentrations of $CaCl_2$ were added to trigger the contractions. The maximal tension attained at 3 mM Ca^{2+} was considered as 100%. Each point shows the mean and vertical lines show s.e.mean ($n=6$).

Effects of neurotoxins on high potassium-induced contraction

The effects of neurotoxins on potassium-induced contraction of rat spleen were expressed as EC_{50} ratios that were calculated from EC_{50} values of potassium-induced contractions in the presence or absence of neurotoxins. Tetrodotoxin (TTX), ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC,

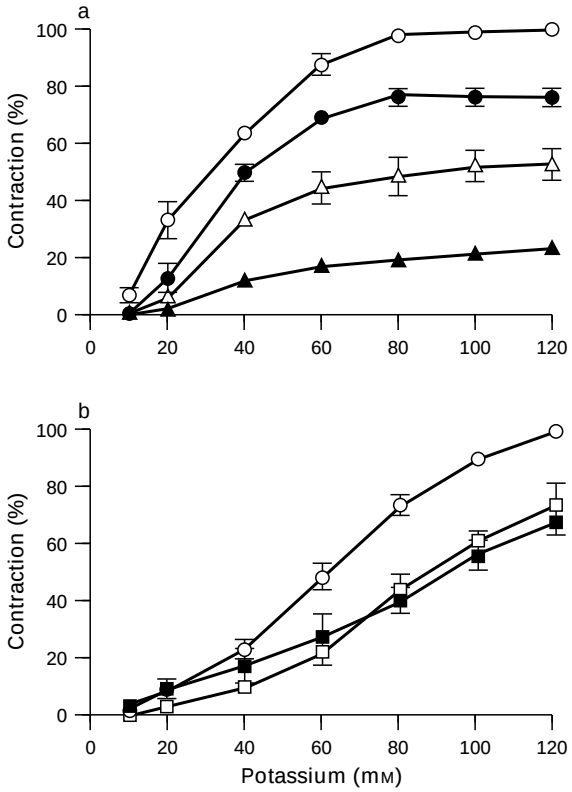

Figure 3 Effects of nifedipine on the high potassium-induced contraction in rat aorta (a) and spleen (b). Rat aorta or spleen was preincubated with DMSO (0.1%, ○) or different concentrations of nifedipine (●, △, ▲, □ and ■ representing 0.003, 0.01, 0.03, 1 and 10 μM, respectively) at 37°C for 15 min, then potassium was added cumulatively to trigger the contraction. The magnitude of the maximal contraction achieved in the control group was taken as 100%. Each point represents the mean and vertical lines show s.e.mean ($n=6$).

Table 1 Inhibition of high potassium-induced contractions in rat spleen and thoracic aorta by organic and inorganic Ca^{2+} channel antagonists

	Rat spleen (IC_{50} value)	*Rat aorta* (IC_{50} value)	*IC_{50} ratio* (spleen/aorta)
Nifedipine (μM)	>10	0.014±0.003	>714
Verapamil (μM)	40.6±3.0	0.14±0.02	294
Diltiazem (μM)	35.7±8.0	0.47±0.03	77
Cadmium (mM)	0.49±0.01	0.013±0.001	38
Nickel (mM)	5.6±0.3	0.9±0.1	6.3
Cobalt (mM)	>10	1.4±0.1	>7.1
R-(+)-Bay K8644 (μM)	27.4±1.4	0.48±0.09	58
Pimozide (μM)	9.3±1.0	3.6±0.7	2.6

IC_{50} ratio was calculated from the IC_{50} value of each antagonist in rat spleen and aorta. IC_{50} value and IC_{50} ratio are expressed as means±s.e.mean ($n=5-6$). The IC_{50} value of each inhibitor on high potassium-induced contraction in rat spleen is significantly different from that value in rat aorta ($P<0.001$).

at a concentration of 1 μM, had no effect on potassium-induced contraction of rat spleen; their EC_{50} ratios were about 1 (Table 3). Furthermore, combined use of ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC did not cause a more pronounced inhibition of potassium-induced contraction of rat spleen (Table 3).

Table 2 Relaxing effects of organic and inorganic Ca^{2+} channel antagonists on potassium (80 mM)-induced tonic contraction in rat spleen and thoracic aorta

	Rat spleen (EC_{50} value)	*Rat aorta* (EC_{50} value)	*EC_{50} ratio* (spleen/aorta)
Nifedipine (μM)	>30	0.08 ± 0.02	>375
Verapamil (μM)	41.8 ± 10.8	0.48 ± 0.13	87.1
Diltiazem (μM)	75.8 ± 9.9	0.84 ± 0.18	90.2
Cadmium (mM)	1.3 ± 0.4	0.04 ± 0.01	32.3
Nickel (mM)	>10	1.3 ± 0.2	>7.5
Cobalt (mM)	>10	0.54 ± 0.16	>18.5

EC_{50} value was calculated from the relaxation concentration-response curve of each antagonist. EC_{50} ratio was calculated from the EC_{50} value of each antagonist in rat spleen and aorta. Data are expressed as means$\pm$s.e.mean ($n=5-6$). The EC_{50} value of each inhibitor on high potassium-induced contraction in rat spleen is significantly different from that value in rat aorta ($P<0.01$).

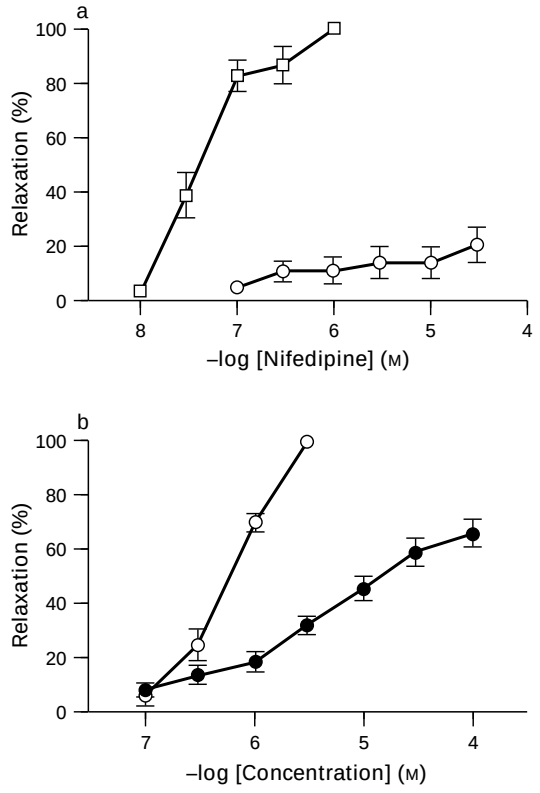

Figure 4 Effects of nifedipine, forskolin and sodium nitroprusside on the sustained contraction induced by potassium in rat spleen and aorta. In (a), rat spleen (○) or aorta (□) was precontracted with potassium (80 mM) for 5 min, then nifedipine was added cumulatively to induce muscle relaxation. In (b), rat spleen was precontracted with potassium (80 mM) for 5 min, then forskolin (○) or sodium nitroprusside (●) was added cumulatively to induce relaxation. The magnitude of contraction before the addition of nifedipine, forskolin or sodium nitroprusside was taken as 100%. Each point represents the mean and vertical lines show s.e.mean ($n=6$).

Effects of various receptor antagonists and inhibitors on potassium-induced contraction in rat spleen

A variety of receptor antagonists and inhibitors were also used to evaluate the mechanism of high potassium-induced contraction in rat spleen. As shown in Table 3, the adrenoceptor, muscarinic, histamine, 5-hydroxytryptamine, angiotensin II, thromboxane, purinoceptor, N-methyl-D-aspartate (NMDA) and non-NMDA, neurokinin and cholecystokinin receptor antagonists did not affect the high potassium-induced contraction in rat spleen. Furthermore, inhibitors of phospholipase C (PLC), protein kinase C (PKC), cyclo-oxygenase, lipoxygenase, the Na^+-Ca^{2+} exchanger, cytochrome *P*-450, mechanosensitive Ca^{2+} channels and the intracellular Ca^{2+} release from the sarcoplasmic reticulum also had no effect on high potassium-induced contraction in rat spleen. In contrast, chlorpromazine (10 μM) slightly inhibited the high potassium-induced contraction in rat spleen (Table 3). Forskolin and nitroprusside which activate adenylate and guanylate cyclases respectively, also caused concentration-dependent relaxations of high potassium (80 mM)-induced contraction of rat spleen with EC_{50} values of 0.55 ± 0.04 and 20.0 ± 2.7 μM, respectively (Figure 4b).

Discussion

The transmembrane influx of extracellular Ca^{2+} through specific calcium channels is now accepted to have an important role in the excitation-contraction coupling of smooth muscle (Bolton, 1979). High potassium activates the voltage-operated Ca^{2+} channel (VOC) to induce Ca^{2+} influx and contraction in smooth muscle (Andrew *et al*., 1986). In this study, high potassium induced a contraction that was dependent on both extracellular potassium and Ca^{2+} concentrations in rat spleen. Omission of Ca^{2+} from the Krebs solution abolished, whereas readdition of Ca^{2+} restored, the contraction. In Ca^{2+}-free high potassium (80 mM) solution, Ca^{2+} caused a contraction of rat spleen in a concentration-dependent manner. Moreover, the inorganic Ca^{2+} channel blockers, Ni^{2+}, Co^{2+} and Cd^{2+}, which have proven useful in assessing the general involvement of VOC in a given process (Olivera *et al*., 1994) also inhibited high potassium-induced contraction of rat spleen. Thus, the high potassium-induced contraction of rat spleen is mediated by Ca^{2+} influx through VOC.

One low-voltage activated (T-type) and six high-voltage activated (L-, N-, O-, P-, Q- and R-types) Ca^{2+} channels have been distinguished on the basis of their pharmacological and electrophysiological properties and from results of molecular cloning studies (Zhang *et al*., 1993; Olivera *et al*., 1994). High potassium may not activate the T-type Ca^{2+} channel since it is activated at negative potentials and then inactivated after small changes of membrane potential. Among the high-voltage activated channels, the L-type Ca^{2+} channel is blocked by the 1,4-dihydropyridines, phenylalkylamines and benzothiazepines (Hess, 1990). The identification and purification of peptide toxins help to discriminate between subtypes of dihydropyridine-resistant Ca^{2+} channels (Olivera *et al*., 1994). Among them, ω-conotoxin GVIA and ω-agatoxin IVA selectively block N- and P-type Ca^{2+} channels, respectively, whereas ω-conotoxin MVIIC is a blocker of N- and OPQ-type Ca^{2+} channels (Olivera *et al*., 1985; Hillyard *et al*., 1992; Monje *et al*., 1993; Minz *et al*., 1992). The high potassium-induced contraction of rat spleen was not affected by these neurotoxins indicating that the contraction was not mediated by Ca^{2+} influx through N-, O-, P-, Q- and R-types Ca^{2+} channels. Since the N-, P-, Q- and R-types Ca^{2+} channels are mainly distributed to neurones and participate in neurotransmitter release, these results also imply that high potassium-induced contraction in rat spleen is not mediated by release of neurotransmitter(s) from nerve terminals. Furthermore, the L-type Ca^{2+} channel blockers inhibited and relaxed high potassium-induced contraction of rat spleen only at concentrations much

Table 3 Effects on high potassium-induced contraction of various receptor antagonists and inhibitors in rat spleen

Treatment	*EC_{50} ratio*	*Selectivity*
Tetrodotoxin (1 μM)	1.21 ± 0.08	Na^+ channel
ω-Conotoxin GVIA (1 μM)	1.11 ± 0.14	N-type Ca^{2+} channel
ω-Conotoxin MVIIC (1 μM)	1.62 ± 0.07	N- and OPQ-type Ca^{2+} channels
ω-Agatoxin IVA (1 μM)	1.03 ± 0.03	P-type Ca^{2+} channel
ω-Conotoxin GVIA (1 μM) + ω-Conotoxin MVIIC (1 μM) + ω-Agatoxin IVA (1 μM)	1.61 ± 0.24	
Prazosin (1 μM)	1.00 ± 0.01	α_1-Adrenoceptor
Chloroethylclonidine (10 μM)	1.13 ± 0.10	α_{1B}-Adrenoceptor
Yohimbine (10 μM)	1.15 ± 0.05	α_2-Adrenoceptor
Propranolol (10 μM)	0.98 ± 0.08	β-Adrenoceptor
Atropine (1 μM)	1.04 ± 0.05	Muscarinic receptor
Diphenhydramine (10 μM)	1.12 ± 0.05	H_1 histamine receptor
Cimetidine (10 μM)	0.97 ± 0.09	H_2 histamine receptor
Ketanserin (10 μM)	1.66 ± 0.18	5-HT_2/5-HT_{1C} receptor
3-Tropanyl-indole-3-carboxylate (10 μM)	0.85 ± 0.07	5-HT_3 receptor
Saralasin (3 μM)	1.24 ± 0.13	Angiotensin II receptor
GR32191B (10 μM)	1.10 ± 0.04	Thromboxane receptor
Domperidone (10 μM)	1.23 ± 0.07	Peripheral dopamine receptor
Chlorpromazine (10 μM)	3.11 ± 0.65	Dopamine receptor
Naloxone (10 μM)	1.12 ± 0.05	Opioid receptor
Suramin (10 μM)	1.29 ± 0.17	P_{2x} and P_{2y}-purinoceptor
(±)-2-Amino-5-phosphonopentanoic acid (10 μM)	1.20 ± 0.17	NMDA receptor
DNQX (10 μM)	0.88 ± 0.11	Non-NMDA receptor
L-659,877 (10 μM)	1.08 ± 0.07	NK_2 tachykinin receptor
L-703,606 (10 μM)	1.33 ± 0.08	NK_1 tachykinin receptor
Lorglumide (10 μM)	1.06 ± 0.04	CCK receptor
PD135,158 N-methyl-D-glucamine (10 μM)	1.18 ± 0.05	CCK_B receptor
Benextramine (10 μM)	1.38 ± 0.12	α_1- and α_2-Adrenoceptor
Neomycin (5 mM)	1.17 ± 0.06	Phospholipase C
Staurosporine (100 nM)	1.44 ± 0.12	Protein kinase C
Indomethacin (10 μM)	1.26 ± 0.14	Cyclo-oxygenase
Nordihydroguaiaretic acid (10 μM)	1.50 ± 0.13	Lipoxygenase/cyclo-oxygenase
Amiloride (500 μM)	1.40 ± 0.05	Na^+-Ca^{2+} exchanger
Dantrolene (30 μM)	1.40 ± 0.18	Intracellular Ca^{2+} release
TMB-8 (30 μM)	1.30 ± 0.03	Intracellular Ca^{2+} release
Econazole (10 μM)	1.30 ± 0.03	Store-regulated Ca^{2+} influx
Gadolinium (500 μM)	1.01 ± 0.05	Stretch-activated Ca^{2+} channel

Dimethylsulphoxide (0.1%, control) or inhibitors were preincubated with spleen for 15 min except for chloroethylclonidine which was preincubated for 30 min. EC_{50} ratios were calculated from EC_{50} values of potassium-induced contraction of rat spleen in the presence or absence of inhibitors and expressed as means ± s.e.mean ($n = 6$).

higher than those used to block high potassium-evoked contraction of rat aorta. Indeed, nifedipine inhibited and relaxed high potassium-induced contraction of rat spleen by not more than 40%. Thus, high potassium may activate a VOC in rat spleen that is pharmacologically different from the dihydropyridine-sensitive L-type Ca^{2+} channel existing in vascular smooth muscle cells.

In addition to VOC, there are a number of mechanisms for Ca^{2+} entry into the cytoplasmic compartment of smooth muscle cells. Agonists, but not changes in membrane potential, can activate receptor-operated Ca^{2+} channels (ROC) through the intermediate action of a second messenger. Although, the nature of the second messenger that links the receptors to ROC is still unresolved, involvement of G proteins and phospholipase C in second messenger activation of ROC has been suggested (van Breemen, 1989). Diacylglycerol, one of the products of phospholipase C triggered phosphatidylinositide breakdown, activates the enzyme protein kinase C. Protein kinase C may, in turn, enhance L-type Ca^{2+} channel activity via phosphorylation (Galizzi *et al*., 1987). Mechanical factors such as stretch, may also stimulate a stretch-activated cation channel leading to the influx of Ca^{2+} into smooth muscle cells (Lansman *et al*., 1987). Moreover, Na^+-Ca^{2+} exchange that occurs in response to increases in intracellular Na^+ activates the entry of Ca^{2+} into the cytoplasm of smooth muscle cells (Hathaway *et al*., 1991). Neomycin, staurosporine, Gd^{3+} and amiloride which inhibit phospholipase C (PLC), protein kinase C (PKC), stretch-activated cation channel and Na^+-Ca^{2+} exchange, respectively, had no effect on high potassium-induced contraction of rat spleen. These results indicate that high potassium-stimulated Ca^{2+} influx in rat spleen is not mediated by either PLC, PKC, the stretch-activated cation channel or the Na^+-Ca^{2+} exchanger.

Many physiological agonists act by increasing the $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores. Recently, it has been suggested that the emptying of the intracellular Ca^{2+} stores could cause an increase in plasma membrane Ca^{2+} (and Mn^{2+}) permeability in several types of cells (Takemura *et al*., 1989; Jacob, 1990; Sage *et al*., 1990a, b). The signal that relates levels of Ca^{2+} in the stores to an increase in plasma membrane permeability to Ca^{2+} has not been elucidated, but a number of mechanisms have been proposed (Vostal *et al*., 1991; Alvarez *et al*., 1991; Irvine, 1991; Neher, 1992). However, econazole, the imidazole antimycotic compound that has been shown to inhibit store-regulated Ca^{2+} influx by interaction with plasma membrane Ca^{2+} channels (Vostal & Fratantoni, 1993), did not affect the high potassium-induced contraction of rat spleen. Moreover, dantrolene and TMB-8, two inhibitors of intracellular Ca^{2+} release (van Winkle, 1976; Ishihara & Karaki, 1991), also had no effect on high potassium-induced contraction of rat spleen. Thus, intracellular Ca^{2+} mobilization and store-regulated Ca^{2+} influx are not involved in this potassium-induced contraction.

Isolated strips of spleen from rat have been used *in vitro* to

study the role of α_1- and α_2-adrenoceptors in the contraction of spleen, in response to stimulation (Ahmed & Naylor, 1982). In addition, Felten *et al*. (1985) showed that neuropeptide Y-like, Met-enkephalin-like, cholecystokinin (CCK)-like and neurotensin-like immunoreactivity are present in the splenic white pulp. In a more recent study, Elenkov & Vizi (1991) suggested that the release of noradrenaline is subjected to presynaptic modulation through different presynaptic receptors and this modulation plays a role in the communication between the central nervous system and the spleen. The possibility that various neurotransmitters and neurohormones might participate in potassium-induced contraction of rat spleen was evaluated. As shown in Table 3, it is clear that they are not involved in potassium-induced contraction of rat spleen since TTX and a variety of receptor antagonists did not affect the contraction. This notion is also supported by the finding that neurotoxins inhibit neuronal release of neurotransmitters but not the high potassium-induced contraction of rat spleen. However, chlorpromazine at a concentration of 10 μM slightly inhibited the high potassium-induced contraction of rat spleen. Chlorpromazine is a calmodulin antagonist (Levin & Weiss, 1979). It is well known that Ca^{2+} regulation of smooth muscle contraction is mediated by a light chain kinase activated by calmodulin in the presence of Ca^{2+}. Activation of myosin light chain kinase increases the activity of actomyosin ATPase leading to the contraction of actomyosin system. Thus, chlorpromazine may relax the high potassium-induced contraction of rat spleen by inhibiting calmodulin-dependent myosin light chain phosphorylation and thereby suppressing the actin-myosin interaction and concomitant myosin ATPase activation.

In conclusion, the high potassium-stimulated contraction of rat spleen may be mediated by a novel and pharmacologically uncharacterized voltage-operated Ca^{2+} channel. Further investigation of its single channel properties, distribution and possible physiological role is warranted.

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