BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 May; 17 (3): 197 - 201

Original Research

Protein kinase C plays no role in KCl-induced vascular contraction in Ca^{2+}-free medium

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KEY WORDS calcium; protein kinase C; Calphostin C; phorbol esters; vascular smooth muscle

AIM: To examine the role of protein kinase C (PKC) on the sustained contractile responses of rat aorta to high KCl in isotonic Ca^{2+}- and Mg^{2+}-free solutions. **METHODS:** The effects of phorbol 12-myristate 13-acetate (PMA, a PKC activator) and Calphostin C (a selective PKC inhibitor) were observed on the sustained contraction of rat aorta induced by K^+ 136 mmol·L^{-1}. EGTA (100 μmol·L^{-1}) was added to prepare the Ca^{2+}-free medium and EDTA (100 μmol·L^{-1}) was added to reduce or remove the Mg^{2+}. **RESULTS:** Aortic contraction to KCl was prominent in low Mg^{2+} medium and was enhanced by EDTA (K-EDTA contraction). Such contraction was concentration-dependently inhibited by Mg^{2+}, but was not affected by Calphostin C 1 μmol·L^{-1}. Pretreatment of the aortic preparations with PMA (0.8 μmol·L^{-1}) potentiated the contraction to KCl in Ca^{2+}-free, low Mg^{2+} medium and higher concentration of Mg^{2+} was required to cause relaxation. Such a reduced sensitivity to Mg^{2+} in the presence of PMA was partially reversed by Calphostin C and was accompanied by an increased sensitivity to Ca^{2+}, which concentration-dependently caused contraction following Mg^{2+}-induced relaxation. However, in the presence of EDTA 100 μmol·L^{-1} (eg, Mg^{2+}-free medium), the maximal contraction to KCl in Ca^{2+}-free medium was not affected by PMA or Calphostin C. **CONCLUSION:** KCl-induced contraction in Ca^{2+}-free and Mg^{2+}-free + EDTA 100 mmol·L^{-1} medium was not affected by PMA or Calphostoin C, indicating that PKC plays no role in such contractile responses.

As in most cells, the resting membrane potential of smooth muscle cells arises primarily from the electrochemical gradient of the potassium ions (K^+). Therefore, the initial step leading to the contractile response to KCl is the membrane depolarization of the smooth muscle cell[1,2], followed by the opening of voltage-operated Ca-channels (VOC) resulting in Ca^{2+}-entry[3-5]. Studies with organic Ca^{2+}-entry blockers and inorganic Ca^{2+}-antagonists, (such as Ni^{2+}, Mn^{2+}, Cd^{2+}, Mg^{2+}, and La^{3+})[6-8] have reinforced the important role of VOC in the development of K^+-induced contraction. Subsequent to the opening of VOC by high concentrations of K^+ is the elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting from the influx of Ca^{2+}[9-11], which then activates the calmodulin-dependent contractile machinery. Therefore, K^+-induced contraction in smooth muscle is generally believed to be absolutely dependent on extracellular Ca^{2+}.

However, we have recently demonstrated the development of prominent, reproducible and reversible contraction in rat aortic rings in response to high concentrations of KCl in the absence of extracellular Ca^{2+} when the extracellular Mg^{2+} was excluded from the physiological saline solution[12]. This observation implies that sustained contractile responses of vascular smooth muscle to high K^+ does not necessarily require the influx of Ca^{2+}, thus an increase in $[Ca^{2+}]_i$, and that Mg^{2+} may play an important role in the modulation of vascular contraction. It is rather unlikely that the aortic contraction to K^+ in Ca^{2+}- and Mg^{2+}-free medium is a result of Ca^{2+}-release. Indeed, when the intracellular Ca^{2+} stores (primarily the sarcoplasmic

[1] Supported by grants from Hong Kong Research Grant Council CRCG and in part by the Heart and Stroke Foundation of Ontario.
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Received 1995-11-28 Accepted 1996-01-25

reticulum) of the vascular smooth muscle was depleted of its Ca^{2+} by prior treatment with norepinephrine (via the inositol trisphopsphate-sensitive Ca^{2+} release pathway), ryanodine or caffeine (via the Ca^{2+}-induced Ca^{2+} release pathway), or calimycin (via release of Ca^{2+} from all intracellular compartments) in the absence of extracellular Ca^{2+}, subsequent stimulation of the vascular preparation with high K^+ in Ca^{2+}-free and Mg^{2+}-free medium still resulted in the development of tension[12,13]. Therefore, in this work we test the hypothesis that activation of PKC, which is known to increase the sensitivity of the contractile machinery to the low $[Ca^{2+}]_i$[14], contributes to the sustained contractile responses to KCl in rat aorta in Ca^{2+}- and Mg^{2+}-free medium. Indeed, K^+-stimulation has been reported to be associated with activation of PKC in some cells[15].

MATERIALS AND METHODS

Sprague-Dawley rats (♂, 250 - 300 g) were stunned and decapitated following the guideline of the government and university regulations on the use of experimental animal. Thoracic aortic rings were placed in modified physiological saline solution (PSS) at pH 7.2 containing ($mmol \cdot L^{-1}$): NaCl 120, KCl 2.5, $NaHCO_3$ 12.5, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11, and HEPES-Tris 10 at room temperature (23 - 24 ℃) bubbled with 95 % O_2 and 5 % CO_2. The experiment were performed at the room temperature in order to keep the magnitude of the contraction to PMA sufficiently low to allow further development of contraction upon introduction of KCl in Ca^{2+}-free medium. Aortic rings were mounted isometrically under an optimum resting tension of 1.5 - 1.8 g for 60 min equilibration. Recording of contractile force was monitored using a force displacement transdusers (Glass F-10) coupled to a Grass model 7H polygraph recorder.

Besides PSS, the following solutions were also used: 1) K-PSS: same as PSS except that Na + was totally replaced by K^+ (eg, K^+ 136 $mmol \cdot L^{-1}$); 2) Ca^{2+}-free Na-PSS: same as PSS except that $CaCl_2$ was excluded 3) Ca^{2+}-free low Mg^{2+} K-PSS: same as K-PSS except that $CaCl_2$ was excluded and Mg^{2+} 20 $\mu mol \cdot L^{-1}$. All Ca^{2+}-free solutions contained egtazic acid (EGTA) 100 $\mu mol \cdot L^{-1}$ to chelate the contaminating Ca^{2+} (3 - 10 $\mu mol \cdot L^{-1}$) in the nominally Ca^{2+}-free medium All solutions were prepared with double-distilled and deionized water and were maintained at pH 7.2 For media containing EDTA and/or EGTA, the reported concentrations of Ca^{2+} or Mg^{2+} represent the final free cation concentrations calculated using reported stability constants for the corresponding complexes.

RESULTS

Effects of PMA on aortic contraction in PSS and K-PSS containing edetic acid (EDTA) Aortic rings elicited a weak tension development when the PSS(or Na-PSS) containing Mg^{2+} 1.2 $mmol \cdot L^{-1}$ was replaced by PSS containing high K^+ (136 $mmol \cdot L^{-1}$) and low Mg^{2+} (20 $\mu mol \cdot L^{-1}$). Addition of EDTA 100 $\mu mol \cdot L^{-1}$ chelate the residual Mg^{2+} and perhaps some membrane-bound Mg^{2+} resulted in further enhancement of the contraction. This contraction is referred to as K-EDTA contraction for simplicity (Fig 1).

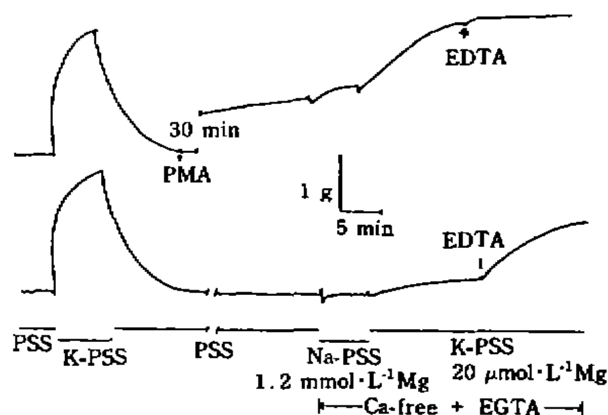

Fig 1. KCl-induced contractions of PMA (0.8 $\mu mol \cdot L^{-1}$)-treated (upper tracing) and control (lower tracing) rat aortic rings in isotonic Ca^{2+}-free low Mg^{2+} (20 $\mu mol \cdot L^{-1}$) solution at room temperature (23 - 24 ℃), pH 7.2. These tracings are typical of more than 4 experiments.

When PMA was added following the control response to K-PSS, tension was gradually developed in PSS, Na-PSS and was more prominently seen in K-PSS containing low Mg^{2+}. Although subsequent addition of EDTA at the plateau phase of the contraction had little effect on its magnitude, K^+-induced contraction (without EDTA) in K-PSS containing low Mg^{2+} was inhibited by Mg^{2+} in a concentration-dependent manner. The Mg^{2+}-dependent inhibition profiles of K^+-induced contraction obtained in the presence and absence of PMA treatment revealed a drastic difference in the sensitivity to Mg^{2+}. The concentration of Mg^{2+} causing 50 % inhibition was about 300 and 30 $mmol \cdot L^{-1}$ for the contraction in Ca^{2+}-free K-PSS with

and without PMA treatment, respectively. It seems that PMA treatment caused a decrease in the binidng of Mg^{2+} to the external side of the smooth muscle cell membranes (Fig 2).

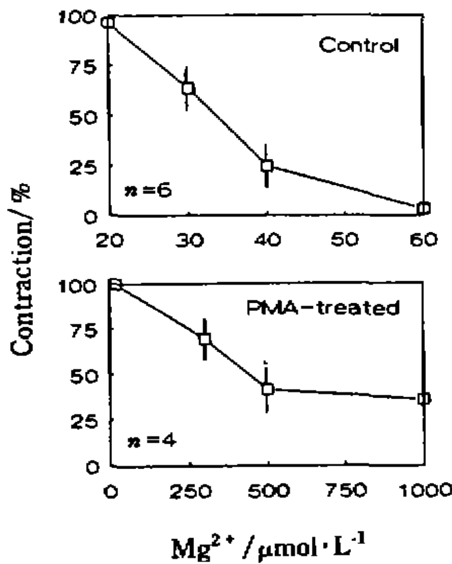

Fig 2. Inhibition of KCl-induced contraction of control and PMA-treated aortic rings in Ca^{2+}-free low Mg^{2+} solution by added Mg^{2+}. The contraction is expressed as % of tension developed in Ca^{2+}-free low Mg^{2+} K-PSS. $\bar{x} \pm s$.

Effects of PMA on Ca^{2+}-induced contraction

PMA treatment of the rat aorta caused a left-shift of the concentration of Ca^{2+} required to induced 50 % of the maximal contraction (from about 300 μmol·L^{-1} in the control to about 80 μmol·L^{-1} in PMA-treated preparations). Ca^{2+}-induced contraction was studied *after relaxation by 0.05 mmol·L^{-1} Mg^{2+} of the K-*EDTA contracted aortic rings. The contraction is expressed as % of tension developed in K-PSS following additon of 0.1 mmol·L^{-1} EDTA (Fig 3).

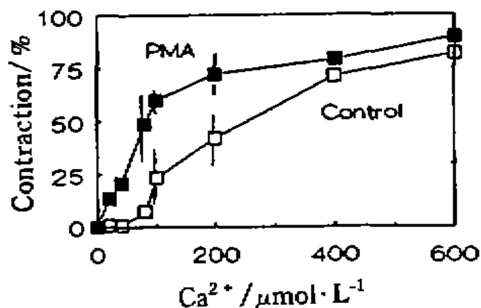

Fig 3. Effect of Ca^{2+} on control and PMA (0.8 μmol·L^{-1})-treated aortic rings. $n=4$, $\bar{x} \pm s$.

Effects of Calphostin C on K-EDTA contraction in Ca^{2+}-free medium K-EDTA contraction in Ca^{2+}-free medium was not affected at all by Calphostin C (1 μmol·L^{-1}). Although Calphostin C partially inhibited the tension development in Ca^{2+}-free low Mg^{2+} K-PSS, it had no effect on the magnitude of the contracton induced by K-EDTA in Ca^{2+}-free medium (Fig 4).

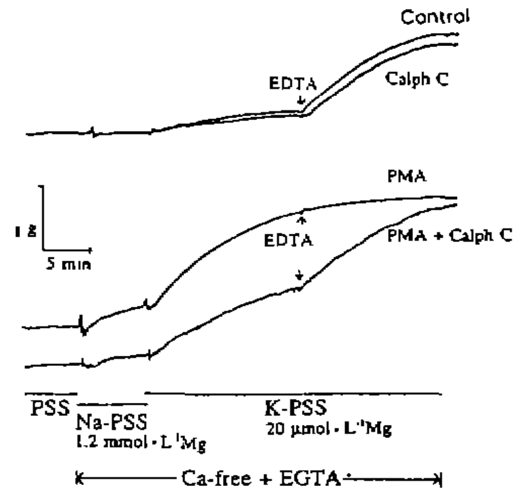

Fig 4. Effect of Calphostin C (1 μmol·L^{-1}) on K^+- and K-EDTA contractions in control (upper tracings) and PMA-treated (lower tracings) aortic rings. Calphostin C was added to the medium before PMA (0.8 μmol·L^{-1}) and remained for 20 min. The tracings are typical of 4 experiments.

DISCUSSION

We have previously demonstrated[12,13] that rataortic rings developed reproducible contractions in Ca^{2+}-free, nominally Mg^{2+}-free, Na^+-free, K^+-rich isotonic solution, but not in Ca^{2+}-free, nominally Mg^{2+}-free, K^+-free, Na^+-rich isotonic solution. Further reduction of the residual Mg^{2+} by EDTA in the above solutions potentiated this KCl-induced contraction and allowed the development of a relatively small contraction in Na^+-rich medium, respectively. The amplitude of K-EDTA contraction was almost the same as the amplitude of contraction induced in K^+-rich (K^+ 136 mmol·L^{-1}), Ca^{2+}-containing (2.5 mmol·L^{-1}) solution. Such KCl-induced contraction was completely inhibited by 3 mmol·L^{-1} Mg^{2+} (also by 3 mmol·L^{-1} Ni^{2+} or Cd^{2+}). Nifedipine (1 μmol·L^{-1}) also inhibited

such K-EDTA contraction in Ca^{2+}-free solution. Modulators of Ca^{2+} in sarcoplasmic reticulum (ryanodine, caffeine and norephinephrine) and Ca^{2+}-ionophore (calimycin) had no effect on this K-EDTA contraction[13]. Based on these findings, it was suggest that KCl-induced contraction in rat aorta is not dependent on the increase in $[Ca^{2+}]_i$ following membrane depolarization and is not a result of the release of Ca^{2+} from intracellular stores. We propose that the competition of K^+ for Mg^{2+} and Na^+ at external binding sites on the plasma membranes of the aortic smooth muscle cells is primarily responsible for the development of vascular contraction.

On the other hand, protein kinase C has frequently been implicated in the experimental observations in which the contractile response is dissociated from the elevation of $[Ca^{2+}]_i$, since activation of PKC may lead to increased sensitivity of the contractile machinery to cytosolic Ca^{2+}. We have employed two different approaches to investigate into such a hypothesis that activation of PKC is involved in K-EDTA contaction in Ca^{2+}- and Mg^{2+}-free medium: the use of PKC activator, PMA and its inhibitor, Calphostin C. Our results showed that pretreatment of rat aortic rings with PMA indeed caused gradual tension development in Ca^{2+}-free K^+-rich PSS containing low Mg^{2+} (20 $\mu mol \cdot L^{-1}$ Mg^{2+}) and this contraction was inhibited concentration-dependently by added Mg^{2+} or further activated by added Ca^{2+}. Indeed, PMA treatment resulted in decreased sensitivity to Mg^{2+}, which was also accompanied by increased sensitivity to externally added Ca^{2+}. However, upon the removal of the residual Mg^{2+} (including plasma membrane-bound Mg^{2+}) with 0.1 $mmol \cdot L^{-1}$ EDTA, the same maximal contraction was observed in the control (no PMA treatment), PMA-treated and PMA-treated plus Calphostin C groups. Therefore, K-EDTA contraction in Ca^{2+}- and Mg^{2+}-free medium was not affected by the presence of either the PKC activator, PMA, or its inhibitor, Calphostin C. These results, together with our earlier findings[13], prompt us to conclude that K-EDTA contraction or rat aorta in Ca^{2+}-free medium does not require Ca^{2+}-influx, does not involve release of Ca^{2+} from intracellular stores and is not due to the activation of PKC. It appears that K-EDTA contraction is closely associated with the change of the surface charge of the plasma membrane as a result of competition of physiologically relevant cations at either side of the smooth muscle plasma membranes.

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蛋白激酶C不参与在无钙溶液内高钾引起的血管收缩

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关键词 钙; 蛋白激酶C; Calphostin C; 咐呼醇酯类; 血管平滑肌

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1996 May; 17 (3): 201 - 206

Suppression of channel conductance by diacetyl monoxime in guinea pig and embryonic chick cardiomyocytes

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KEY WORDS oximes; diacetyl monoxime; myocardium; cultured cells; calcium channels; sodium channels; potassium channels; patch-clamp techniques

AIM: To examine the effects of diacetyl monoxime (DAM), and putative dephospohrylating agent, on conductances of the cardiac Ca, Na, and K channels. **METHODS**: The Ca (I_{Ca}), Na (I_{Na}), and K (I_K) currents were recorded in single ventricular myocytes from guinea pigs and chick embryos before and after addition of DAM using the whole-cell voltage-clamp technique. **RESULTS**: DAM 10 mmol·L^{-1} reduced rapidly the amplitudes of I_{Ca}(by about 30 %), I_{Na}(by about 25 %), and I_K(by 25 % - 50 %) without alterations of the voltage-dependence. **CONCLUSION**: DAM was a channel inhibitor of the unique type having nonselective phosphatase activities.

Diacetyl monoxime (DAM) was used for the treatment of organophosphorus poisoning, in which acetylcholinesterase is reactivated by the oxime[1]. Because of its nucleophilic properties shared with oximes, DAM was suggested to possess a dephosphorylating (phosphatase-like) action, which is evidenced via an interference with cAMP-dependent phosphorylations from the cytoplasmic side[2,3]. Since the activity of the Ca[4], Na[5], or K channel[6] is controlled by phosphorylations of many types and steps, it is of interest whether DAM deactivates such channels through its phosphatase-like properties.

Received 1996-01-02 Accepted 1996-01-22

MATERIALS AND METHODS

Voltage-clamp (v - c) experiments were carried out using ventricular myocytes from chick embryos (cultured for 5 - 60 h) and guinea pigs (dispersed 1 - 6 h before each experiment)[7]. Ventricular myocytes from guinea pigs were obtained[8].

Using the whole-cell v - c method, membrane currents were recorded. Pipettes were Sylgard-coated and heat-polished. The composition of the pipette solution was (mmol ·L^{-1}): K- or Cs-aspartate 130, egtazic acid (EGTA) 11, $CaCl_2$ 1, NaCl 10, $MgCl_2$ 2, Na_2ATP 5, glucose 5, and HEPES 10.0 (pH 7.2). The composition of the bath solution (superfusate) was (mmol·L^{-1}): NaCl 140, CsCl or KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, Na-pyruvate 5, glucose 10, and HEPES 10 (pH 7.4). In experiments of the Ca (I_{Ca}) and K currents (I_K), tetrodotoxin (TTX) 0.01 mmol