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Ca^{2+}-independent contraction induced by hyperosmolar K^+-rich solutions in rat uterus

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Abstract

The present experiments were designed to investigate the mechanisms involved in the contractile responses evoked by KCl, added either isoosmotically or hyperosmotically, in the rat uterus. Exposure of uterine strips to a Ca^{2+}-free, 3 mM EGTA-containing solution abolished the responses induced by isoosmotic KCl solutions. Conversely, addition of hyperosmolar KCl induced concentration-dependent tonic responses in a Ca^{2+}-free, 3 mM EGTA-containing solution. The maximum increase in tension was reached with 210 mM K^+. The response to hyperosmotic K^+ was unaffected by previous depletion of intracellular Ca^{2+} stores with oxytocin (1 μM), by inhibition of refilling of the intracellular Ca^{2+} stores using cyclopiazonic acid (10 μM) or by increasing the concentration of EGTA in the medium to 10 mM. Sucrose and mannitol (60–420 mM) induced concentration-dependent sustained contractions which were not reproducible and were significantly smaller in size than those evoked by the maximally effective concentration of hyperosmotic K^+ (210 mM). The contraction induced by hyperosmotic K^+ in Ca^{2+}-free solution was not altered by the calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 100 μM), the Ca^{2+}/calmodulin protein kinase II inhibitor 1-[*N,O-bis*(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, 10 μM) or the tyrosine kinase inhibitor genistein (10 μM). The protein kinase C inhibitor calphostin C (1–3 μM) failed to modify the K^+-effect curve, which was however partially inhibited in the presence of the non-selective protein kinase inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7, 3-100 μM). The protein kinase inhibitor staurosporine (30–300 nM) depressed the contraction induced by hyperosmolar K^+ in a concentration-dependent manner. The contraction induced by sucrose in Ca^{2+}-free solution was unaffected by W-7 (100 μM) and KN-62 (10 μM) but was partially reduced by calphostin C (1 μM), H-7 (30 μM), staurosporine (100 nM) and genistein (10 μM). These results suggest that different mechanisms are involved in the responses evoked by isoosmotic and hyperosmotic KCl in the rat uterus. A component of the contraction induced by hypertonic KCl seems mainly independent of both external and internal Ca^{2+} and of hyperosmolar stress. This contraction is not mediated by protein kinase C, Ca^{2+}/calmodulin-dependent kinases or protein tyrosine kinases but involves activation of other, at the present unknown, staurosporine-sensitive protein kinase(s).

Keywords: Hyperosmolarity; Protein kinase C; Genistein; Staurosporine; Uterus, rat

1. Introduction

Contraction of smooth muscle can occur through both electromechanical and pharmacomechanical coupling (Somlyo and Somlyo, 1994). In the rat uterus, the contraction induced by maximally effective concentrations of agonists, such as oxytocin and acetylcholine, involves both mechanisms (Lalanne et al., 1984; Edwards et al., 1986; Phaneuf et al., 1993; Kasai et al., 1994) whereas the contraction induced by high K^+ solutions is primarily dependent on electromechanical coupling (Falk, 1991; Ausina et al., 1996).

In most tissues, the KCl-induced contraction is highly sensitive to Ca^{2+} channel blockers and disappears in a Ca^{2+}-free solution, suggesting that it is mainly mediated by Ca^{2+} influx via voltage-sensitive Ca^{2+} channels (D'Ocon et al., 1991). However, recent evidence obtained

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in smooth muscles suggests that, in addition to promoting Ca^{2+} influx, KCl-induced depolarization may also: (1) release intracellular Ca^{2+}, which can contribute to the increase in $[Ca^{2+}]_i$ during electromechanical coupling (Somlyo et al., 1991; Munro and Wendt, 1994); (2) increase the intracellular levels of inositol trisphosphate (IP_3) (Khac et al., 1992). Moreover, contractions induced by hyperosmolar and isoosmolar solutions of KCl may involve activation of different mechanisms, independently of Ca^{2+} influx via Ca^{2+} channels present in the sarcolemma (Nielsen-Kudsk et al., 1992; Low et al., 1994). In this way, Low et al. (1994) demonstrated that hyperosmolar addition of KCl caused Ca^{2+}-independent contraction in the rat and guinea-pig aorta which was mediated, at least partially, by activation of protein kinase C.

We designed the present work to investigate the effect of KCl, added either isoosmotically or hyperosmotically, on the mechanical activity in the oestrogen-primed rat myometrium. We paid special attention to the mechanism responsible for the hyperosmotic KCl-induced contraction. We thus performed experiments both in the presence and in the absence of external Ca^{2+} ions, or in the presence of a variety of protein kinase inhibitors. Hyperosmotic KCl-induced contraction was also compared with those evoked by other hyperosmotic solutions, containing sucrose or mannitol.

2. Materials and methods

2.1. Animals and tissue preparation

Myometrial tissue was obtained from virgin female Wistar rats (200–250 g) pretreated with 17β-oestradiol benzoate (400 $\mu g \cdot kg^{-1}$, i.p.) 24 h before the experiments. The oestrus stage was confirmed by microscopic examination of vaginal smears. Longitudinal strips of uterine smooth muscle (8–10 mm long by 2 mm in width) were prepared and mounted in isolated tissue baths containing 4 ml of Sund's physiological salt solution. The preparations were bubbled continuously with 95% O_2-5% CO_2 and warmed to 32°C. Changes in isometric tension were recorded by means of force-displacement transducers (Grass FT-03) connected to a LETICA amplifier and a ABB GOERZ SE 130 multichannel recorder.

2.2. Solutions

The composition of the reference physiological salt solution used was (mM): NaCl 154; KCl 5.6; $CaCl_2$ 0.54; $MgCl_2$ 0.95; $NaHCO_3$ 5.95 and glucose 2.78 (pH 7.0). A low Ca^{2+} solution was used to avoid development of myometrial spontaneous activity, which has been shown to influence the amplitude of subsequent contractile responses (Lalanne et al., 1984). In some experiments, the concentration of Ca^{2+} in the solution was increased to 2.16 mM. Isoosmotic K^+ solutions of 30, 60 90, 120 and 150 mM were prepared by substituting KCl for NaCl in the physiological solution. The Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding EGTA at appropiate concentrations (0.03, 3 or 10 mM).

2.3. Experimental protocol

The preparations, stretched to the optimal resting force of 0.5 g, were equilibrated for 45 min and then repeatedly stimulated with a maximally effective concentration of acetylcholine (1 mM) until reproducible responses were obtained. The last response served as an internal standard for all experiments. The tissue was then allowed to equilibrate for a further 60 min before addition of cumulative concentrations of isoosmotic or hyperosmotic KCl, sucrose or mannitol. Three cumulative concentration-response curves for one spasmogen in either reference solution or Ca^{2+}-free solution containing 3 or 10 mM EGTA were made with each strip, with an interval of 60 min between curves. Only one spasmogen was tested on each tissue. However, in some experiments we tested the effects of sucrose in isoosmolar K^+ (60 mM), Ca^{2+}-free solution. To assess the effects of hyperosmotic K^+, increasing concentrations of KCl were added to the bath without omitting any other ion. Stated concentrations of hyperosmotic K^+ refer to the KCl added to the bath plus the KCl concentration present in the physiological solution. The osmolarity of some of these solutions was measured with an osmometer (OSMOMAT 030 crioscopic, GONOTEC).

To investigate the possible involvement of intracellular Ca^{2+} in the contractile responses, the uterine strips were incubated in solution containing 2.16 mM Ca^{2+} for 10 min (Ca^{2+}-loading period) then in a Ca^{2+}-free, 3 mM EGTA-containing solution for 4 min before the application of spasmogens. This Ca^{2+}-loading protocol was carried out in the absence or in the presence of cyclopiazonic acid (10 μM) and was repeated with each uterine strip several times, with an interval of 34 min between responses.

Some experiments were carried out to evaluate the ability of several compounds to inhibit responses of uteri to KCl or sucrose in Ca^{2+}-free solution. Cumulative concentration-response curves for the spasmogen were made in the presence of the drug of interest (test tissues) or the corresponding vehicle (control tissues), which was applied 20 min before the spasmogen and was in contact with the preparation during the generation of the response. In previous experiments, we observed that the second curve for hyperosmotic K^+ in Ca^{2+}-free solution was higher than the first one, while no significant differences were observed between the second and third curves. For this reason, the compounds tested or their vehicles were added to the bath after completion of the second curve, which was considered as control. In the case of sucrose, the second and third cumulative concentration-response curves were significantly smaller than the first one or absent.

Hence, only one curve for sucrose in the absence or presence of the compound of interest was made with each preparation.

2.4. Expression and statistical analysis of results

Contractions were measured in mg and expressed as a percentage of the control response to acetylcholine. In the experiments carried out in a Ca^{2+}-free solution, the maximum increase in tension induced by hyperosmotic KCl in the second concentration-response curve or by sucrose in control tissues was considered as 100%. The EC_{50}, i.e., the concentration producing half of the maximal response, was determined using a least-squares linear regression method. All values in the text and tables are expressed as means ± S.E.M. for n number of experiments. Statistical significance of differences between two means was assessed by Student's t-test. Multiple means were compared by one-way analysis of variance. P values of less than 0.05 were considered to represent significant differences.

2.5. Drugs

Acetylcholine hydrochloride, mannitol, nifedipine, cyclopiazonic acid, genistein, 1-[*N*,*O*-*bis*(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7) and staurosporine were from Sigma (St. Louis, MO, USA). Calphostin C was from Sigma and from RBI. Calphostin C, staurosporine, genistein, cyclopiazonic acid and KN-62 were dissolved in dimethyl sulphoxide. Nifedipine and W-7 were dissolved in absolute ethanol and then diluted with deionized water to appropriate concentrations. The final ethanol or dimethyl sulphoxide concentration in the bathing medium was lower than 0.1% and had no effect on mechanical responses. The other agents were dissolved in deionized water.

3. Results

3.1. Effects of KCl, sucrose and mannitol on the mechanical activity in the rat uterus

Fig. 1A shows a typical tracing of the effect induced by cumulative addition of isoosmolar KCl solutions. Application of increasing concentrations (30–150 mM) of isoosmotic K^+ caused complex contractile effects. The maximum tension development (130.1 ± 7.6% of the response

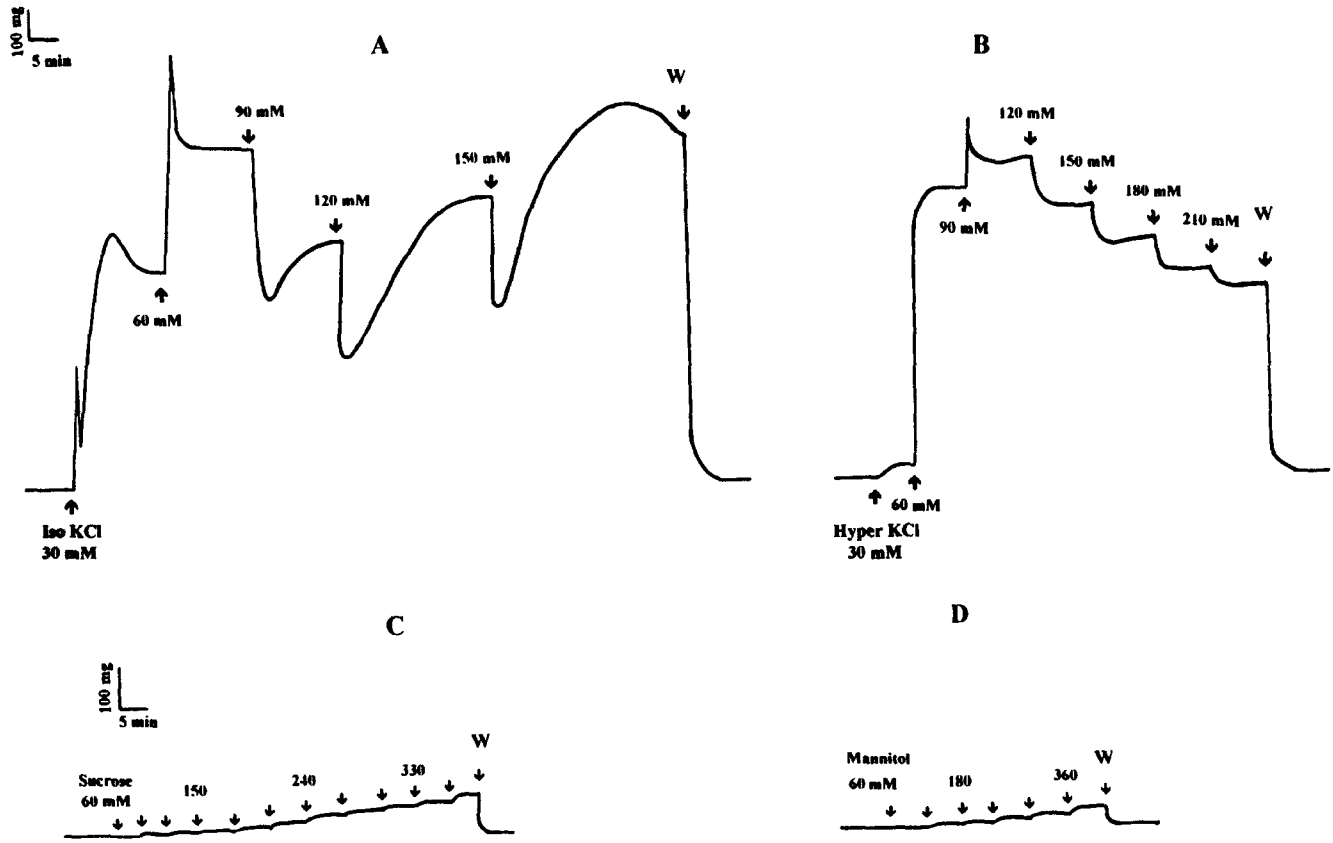

Fig. 1. Representative tracings showing the contractile responses induced by cumulative addition of spasmogens in the rat isolated uterus incubated in Ca^{2+}-containing solution. Traces correspond to the first cumulative concentration-response curve obtained for each stimulant and are representative of typical results of 4–9 experiments.

to acetylcholine, $n = 5$) was achieved with 60 mM K^+ and corresponded to the peak of the transient, phasic component. Second and third control concentration-effect curves for isoosmotic K^+ repeated at 1-h intervals were not significantly different from the initial ones (not shown).

The effect of hyperosmotic K^+ is shown in Fig. 1B. The maximum tension was reached with 90 mM K^+, which induced a phasic contraction (94.8 ± 8.8% of the response to acetylcholine, $n = 9$) followed by a relaxation and a return to a plateau of slightly lower amplitude than the peak tension value. Steady-state tension declined with higher K^+ concentrations (120–240 mM) in a regular, concentration-dependent manner (Fig. 1B). Concentration-response curves for hyperosmotic K^+ were highly reproducible and no significant difference was observed between successive curves obtained with the same uterine strip at 60-min intervals (not shown).

The contractile effect induced by cumulative addition of sucrose or mannitol (60–420 mM) in physiological solution is shown in Fig. 1(C,D). Both agents caused concentration-dependent tonic contractions of a significantly smaller amplitude than those evoked by isoosmotic or hyperosmotic K^+ in similar experimental conditions. The maximal contraction was 5.0 ± 1.8% ($n = 5$) and 3.1 ± 0.7% ($n = 4$) of the maximal acetylcholine-induced contraction for sucrose and mannitol, respectively. The threshold concentration for contraction was 90 mM for both sucrose ($n = 5$) and mannitol ($n = 4$) and the maximum contractile response was achieved with 360 mM for both hyperosmotic agents. When three successive concentration-response curves for sucrose or mannitol were made in the same uterine strip at 60 min intervals, the responses disappeared (in 4 from 10 experiments with sucrose and in 6 from 9 experiments with mannitol) or were significantly smaller than those observed in the first curve. Due to the similarity between the effects induced by sucrose and mannitol, only sucrose was used as an hyperosmotic agent in subsequent experiments.

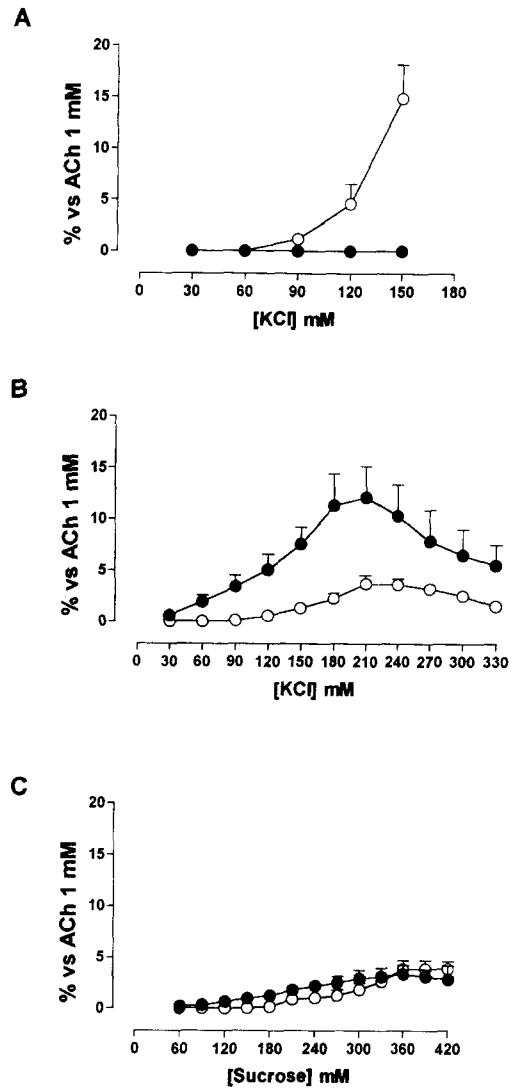

Fig. 2. Cumulative concentration-response curves for (A) isoosmotic K^+ (B) hyperosmotic K^+ and (C) sucrose in oestrogen-dominated rat uterus. Curves were made in the absence of external Ca^{2+} (●) or in Ca^{2+}-containing solution with 1 μM nifedipine (○). Data are the means ± S.E.M. of 4–7 experiments.

3.2. Sensitivity of spasmogen-induced contractions to external Ca^{2+} ions

Pretreatment of strips with nifedipine (1 μM, 20 min) produced marked inhibition of the response to cumulative addition of isoosmolar K^+ solutions. Nevertheless, the rat uterus still contracted in response to higher concentrations of K^+ (90–150 mM), which induced slowly developing tonic contractures of a small amplitude (Fig. 2A). The contraction induced by isoosmotic K^+ at any concentration disappeared after short (4 min) or prolonged (1 h) incubation of the tissue in Ca^{2+}-free, 3 mM EGTA-containing solution (Fig. 2A).

Unlike isoosmotic K^+, nifedipine produced an almost complete abolition of the response to hyperosmotic K^+, although a very small increase in tension was observed for concentrations higher than 90 mM (Fig. 2B). The effect of hyperosmotic K^+ was also markedly reduced after a 1-h incubation in a Ca^{2+}-free, 3 mM EGTA-containing solution, compared to that evoked in the reference physiological solution. However, in Ca^{2+}-free solution, the response to hyperosmotic K^+ at concentrations higher than 60 mM remained greater than that obtained in the presence of nifedipine (Fig. 2B). Concentrations of K^+ higher than 210 mM produced less tension of the uterine strips (Fig. 2B).

The contractile response induced by addition of cumulative amounts of sucrose (60–420 mM) in either physiological solution containing nifedipine 1 μM or Ca^{2+}-free, 3 mM EGTA-containing solution was similar in amplitude to that observed in the reference physiological solution (Fig. 2C). The osmolarity of the different K^+-rich or sucrose-rich Ca^{2+}-free solutions is indicated in the Table 1.

3.3. Sensitivity of spasmogen-induced contractions to intracellular Ca^{2+} ions

To evaluate whether Ca^{2+} can be released from the internal stores in our experimental conditions, we first tested the effects of oxytocin. Application of a maximally effective concentration of oxytocin (1 μM) 4 min after the removal of Ca^{2+} ions induced a transient contraction (14.5 ± 1.1% of the response to acetylcholine in Ca^{2+} (0.54 mM)-containing solution, $n = 16$) followed by a small sustained contraction (5.1 ± 0.4% of the response to acetylcholine, $n = 16$, Fig. 3A,B,C,a). A second application of oxytocin in Ca^{2+}-free solution evoked only the sustained component of the contraction (Fig. 3A,B,C,b).

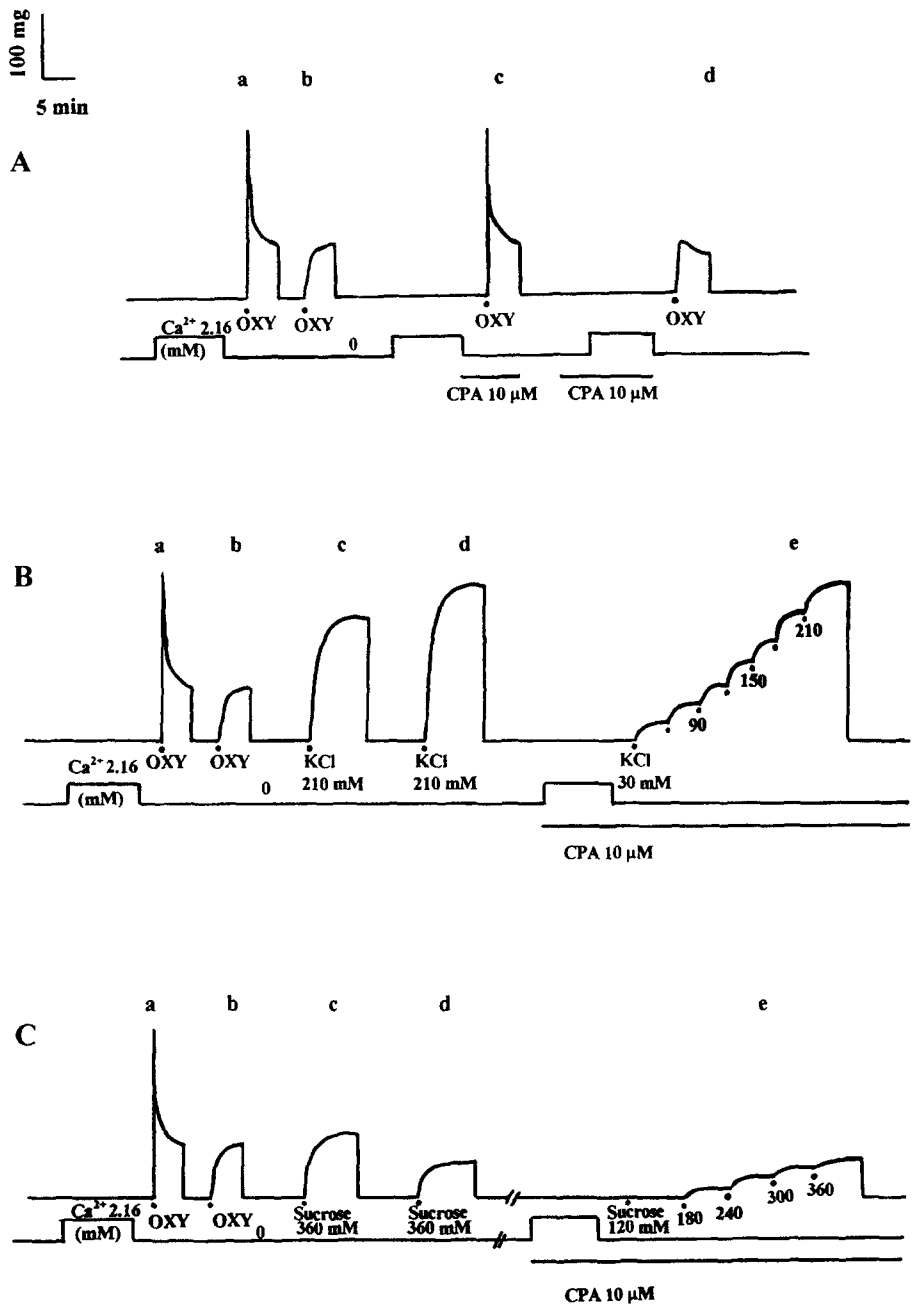

Fig. 3. Representative tracings showing the contractile responses induced by 1 μM oxytocin (OXY), hyperosmotic KCl and sucrose in rat uterine strips after 4 min exposure to a Ca^{2+}-free medium. (A) Oxytocin induced a transient contraction followed by a sustained, small contraction (Aa). When oxytocin was applied a second time in Ca^{2+}-free solution without previous loading of the internal Ca^{2+} stores, only the sustained component was observed (Ab). Cyclopiazonic acid (CPA, 10 μM) did not modify the response to oxytocin when added after Ca^{2+} loading (Ac) but abolished the transient component when added 2 min before and maintained in contact with the preparation during the loading period (Ad). (B,C) Hyperosmolar addition of KCl or sucrose induced slowly developing tonic contractions which were unaffected by depletion of Ca^{2+} stores with oxytocin or by cyclopiazonic acid (c,d,e). Traces are representative of typical results of 5–16 experiments.

The initial transient component was restored after replenishment of the internal Ca^{2+} stores and could be reproduced for several times with the same uterine strip by repeating the Ca^{2+}-loading procedure. This transient contraction was not affected in strips exposed to cyclopiazonic acid (10 μM) after the Ca^{2+}-loading period (Fig. 3Ac) but disappeared when cyclopiazonic acid was added 4 min before the Ca^{2+} loading and was in contact with the preparation until the end of the agonist application (Fig. 3Ad). Moreover, when oxytocin was applied the first time to strips exposed for 1 h to Ca^{2+}-free, 3 mM EGTA-containing solution, only the small tonic contraction was observed. These observations clearly show that only the transient component of the oxytocin-induced response in Ca^{2+}-free solution is dependent on Ca^{2+} release from internal stores.

Cumulative addition of isoosmolar K^+ solutions 4 min after exposure to a Ca^{2+}-free solution containing 3 mM EGTA did not induce any significant contraction (not shown). The lack of response might be due to an additional depletion of intracellular Ca^{2+} stores during the time the curve was being made (aproximately 45 min). For this reason, in some experiments, the increasing concentrations of K^+ were added in a non-cumulative manner, i.e., the muscle strip, exposed to a single isoosmolar solution, was washed and the Ca^{2+}-loading protocol was repeated before addition of the following isoosmolar solution. In these conditions isoosmotic K^+ also failed to produce any contractile effect ($n = 5$, not shown). Conversely, addition of increasing concentrations of hyperosmotic KCl either in a cumulative or non-cumulative manner induced tonic contractions of increasing amplitude, the maximum response being reached with 210 mM K^+. These contractions were unaffected by previous depletion of intracellular stores with oxytocin or by carrying out the Ca^{2+}-loading period in the presence of 10 μM cyclopiazonic acid (Fig. 3B). Similar contractions although of much smaller amplitude were obtained with sucrose, which, independently of the filling state of the Ca^{2+} stores, induced tonic contractions similar to those observed in Ca^{2+}-containing solution ($n = 4$, Fig. 3C).

Table 1
Osmolarity of the Ca^{2+}-free, K^+- or sucrose-rich solutions used in the present study

	Ca^{2+}-free solution	
	NaCl (154 mM) + KCl (5.6 mM)	NaCl (99.6 mM) + KCl (60 mM)
Control	356	350
KCl (mM)		
60	458	–
120	554	–
210	694	–
Sucrose (mM)		
60	431	428
120	510	501
210	588	603
360	796	768

The osmolarity of the solutions is expressed in mosmol/kg H_2O.

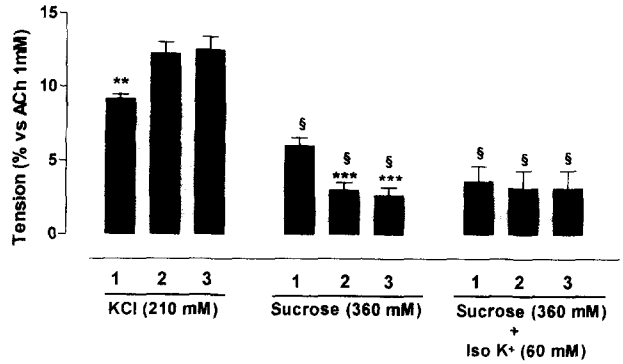

Fig. 4. Maximal contractile responses induced by hyperosmotic KCl and sucrose in rat uterine strips after prolonged exposure to a Ca^{2+}-free medium containing EGTA 3 mM. Three cumulative concentration-response curves were made with each strip with a resting period of 60 min between curves. The maximal responses evoked by spasmogens in the successive curves are expressed as a percentage of the maximal contraction induced by acetylcholine 1 mM in Ca^{2+}-containing solution. Concentrations of the agents inducing maximal contractions are shown in parentheses. Each histogram represent the mean of 6 experiments, with S.E.M. show by vertical bars. ** $P < 0.01$, significantly different from maximal response evoked by KCl 210 mM in the second and third curves; *** $P < 0.001$, significantly different from maximal response evoked by sucrose in the first curve; § $P < 0.001$, significantly different from maximal response to 210 mM KCl in the second curve, one-way ANOVA.

The lack of involvement of intracellular Ca^{2+} in the contractions evoked by hyperosmotic K^+ in a Ca^{2+}-free solution was further evidenced by the observation that the responses were similar when obtained in a Ca^{2+}-free solution containing 10 mM EGTA ($n = 7$). Moreover, three or more successive concentration-response curves for hyperosmotic K^+ could be made with the same uterine strip, exposed to Ca^{2+}-free solution containing either 3 or 10 mM EGTA. In the second curve, hyperosmotic K^+ caused significantly larger contractions than in the initial one while the third and following curves were almost identical (Fig. 4). Successive concentration-response curves for cumulatively added sucrose were also made in Ca^{2+}-free solution containing 3 or 10 mM EGTA. As in reference solution, the responses to sucrose in the second and third curves disappeared or were significantly smaller than those observed in the first curve, although no significant differences were found between mean amplitudes of the second and third curves (Fig. 4). In another set of experiments, sucrose (60–420 mM) was added to isoosmotic KCl (60 mM), Ca^{2+}-free solution containing 3 or 10 mM EGTA. In these conditions, sucrose induced contractions of the same amplitude as those observed in the presence of 5.6 mM KCl (Fig. 4).

3.4. Effects of nifedipine and protein kinase inhibitors

In order to investigate the role of protein kinase C in the contraction induced by hyperosmotic K^+ in Ca^{2+}-free

PSS, we studied the effects of 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine dihydrochloride (H-7, 3–100 μM), staurosporine (30–300 nM) and calphostin C (1–3 μM). As shown in Fig. 5A, H-7 (30 μM) partially reduced the maximum KCl contraction but failed to induce a further inhibition at a significantly higher concentration (100 μM). Staurosporine inhibited the K^+-effect curves in

Table 2
Parameters of the third cumulative concentration-response curves for hypertonically added KCl in rat myometrial strips bathed in Ca^{2+}-free, 3 mM EGTA-containing solution in the absence (control) and presence of W-7, KN-62, genistein and nifedipine

	n	E_{max} (%)	EC_{50} (mM)
Control	6	99.1±2.4	114.0± 6.3
W-7 (100 μM)	6	115.8±3.1	100.4±11.2
KN-62 (10 μM)	4	116.9±7.7	130.7± 6.5
Genistein (10 μM)	8	119.9±5.1	116.8± 3.8
Nifedipine (1 μM)	6	125.6±8.9	132.8± 9.1

Values are means±S.E.M. of n experiments. E_{max} was calculated as a percentage of the maximum increase in tension induced by KCl (210 mM) in the second cumulative concentration-response curve.

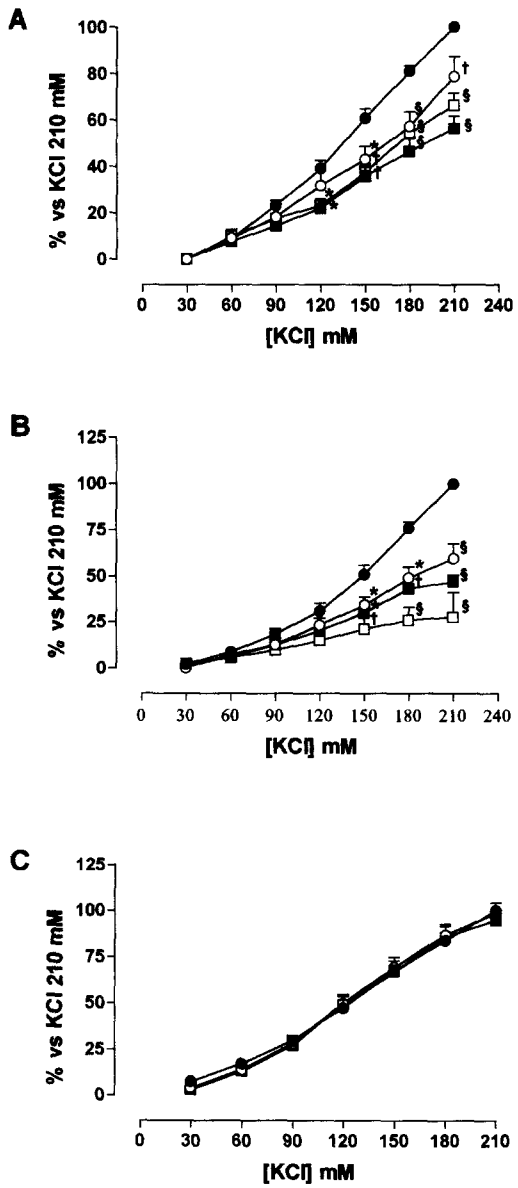

Fig. 5. Cumulative concentration-response curves for hyperosmotic KCl in the absence (●) and in the presence of (A) 3 (○), 30 (■) and 100 μM (□) H-7 ($n=7$); (B) 30 (○), 100 (■) and 300 nM (□) staurosporine ($n=7$–10) and (C) 1 (○) and 3 μM (■) calphostin C ($n=3$–7) in isolated rat myometrium. After 60-min exposure to a Ca^{2+}-free, 3 mM EGTA-containing medium, three concentration-response curves were made with each strip, with an interval of 60 min between curves. Responses were expressed as a percentage of the maximal response induced by KCl 210 mM in the second, control curve. Calphostin C, staurosporine and H-7 were added to the bath 20 min before the third concentration-response curves were made. Data points are means, with S.E.M. show by vertical bars. Statistical differences from control responses: * $P<0.05$; † $P<0.01$; § $P<0.001$; one-way ANOVA.

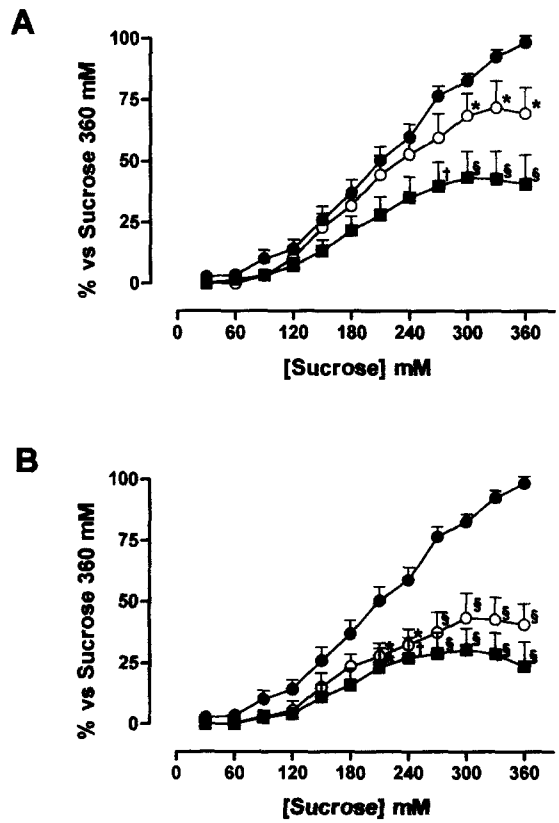

Fig. 6. Effects of protein kinase inhibitors on the cumulative concentration-response curves for sucrose in isolated rat myometrium. One curve was made with each uterine strip, after 60 min exposure to a Ca^{2+}-free, EGTA 3 mM-containing medium. Responses were expressed as a percentage of the maximal response induced by sucrose in control strips. (A) Control (●); staurosporine (100 nM, ○); H-7 (30 μM, ■). (B) Control (●); calphostin C (1 μM, ■); genistein (10 μM, ○). Calphostin C, staurosporine, H-7 and genistein were added to the bath 20 min before the concentration-response curve was made in test tissues. Data points are means for 5–7 experiments, with S.E.M. show by vertical bars. Statistical differences from control responses: * $P<0.05$; † $P<0.01$; § $P<0.001$; one-way ANOVA.

a concentration-dependent manner (Fig. 5B). The responses to hypertonically added K^+ were strongly decreased in the presence of the higher concentration of staurosporine tested (Fig. 5B). The highly selective inhibitor of protein kinase C calphostin C (1 and 3 μM) did not modify the concentration-response curve to hyperosmotic K^+ (Fig. 5C). In another set of experiments, calphostin C (1–5 μM) was added cumulatively to strips precontracted with 210 mM KCl. Again calphostin C was a weak inhibitor: the maximal relaxation reached was $15.4 \pm 8.9\%$ ($n = 3$, $P > 0.05$).

The data summarized in Table 2 show that the concentration-response curves for hypertonically added K^+ were unaffected in the presence of the L-type Ca^{2+} channel blocker nifedipine (1 μM), the calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, 100 μM), the Ca^{2+}-calmodulin-dependent protein kinase II inhibitor 1-[*N,O-bis*(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, 10 μM) or the protein tyrosine kinase inhibitor genistein (10 μM). At the concentrations used in the present study, these inhibitors abolished the sustained component of the response to isoosmotic K^+ (60 mM) in Ca^{2+}-containing solution (not shown).

Staurosporine (100 nM), H-7 (30 μM), calphostin C (1 μM) and genistein (10 μM) significantly reduced the increase in tension produced by cumulative addition of sucrose in a Ca^{2+}-free solution (Fig. 6) while nifedipine (1 μM), W-7 (100 μM) and KN-62 (10 μM) were ineffective (not shown).

4. Discussion

The present study confirms that Ca^{2+} influx through voltage-operated Ca^{2+} channels is the major mechanism involved in the response to isoosmotic or hyperosmotic K^+ in the rat myometrium. However, our results also show that isoosmotic and hyperosmotic KCl-induced contractions markedly differ in their pattern and extracellular Ca^{2+} dependence. This suggests, in agreement with previous reports (Nielsen-Kudsk et al., 1992; Low et al., 1994), that the responses to isoosmotic or hyperosmotic K^+ result, at least in part, from the activation of different mechanisms.

Isoosmotic K^+-induced contractions disappeared in a Ca^{2+}-free medium, demonstrating their dependence on external Ca^{2+}. Pretreatment with the L-type Ca^{2+} channel blocker nifedipine inhibited the contraction induced by the lower concentrations of K^+ (30–60 mM) but did not abolish the response to higher concentrations. Nifedipine has been shown to be the most potent agent among a series of Ca^{2+} channel blockers in the rat uterus (Granger et al., 1986) and, at the concentration used in the present study (1 μM), it is very unlikely that the nifedipine-resistant response involves residual Ca^{2+} influx through voltage-operated Ca^{2+} channels. It must be taken into account that isoosmotic solutions of K^+ were made by substituting NaCl with appropiate concentrations of KCl and, hence, the highest concentration of K^+ was obtained in the almost complete absence of Na^+ in the solution. It is known that exposure of uterine strips to low (60–25 mM) or Na^+-free solutions induces slowly developing contractions which are mediated by Ca^{2+} influx through both L-type Ca^{2+} channels and Na^+/Ca^{2+} exchange (Masahashi and Tomita, 1983; Savineau et al., 1987). These low Na^+ responses are very similar to those observed with 120 and 150 mM K^+ in the present study. It thus appears that the contractions induced by the higher concentrations of isoosmotic K^+ are mainly dependent on Ca^{2+} influx through L-type Ca^{2+} channels but also involve Ca^{2+} entry via Na^+/Ca^{2+} exchange acting in the reverse mode. The last mechanism may be responsible for the small responses which persisted in the presence of nifedipine (Fig. 2A).

The response to cumulative addition of hyperosmotic K^+ almost disappeared in physiological solution containing nifedipine (1 μM). It was therefore surprising to observe that uterine strips exposed to Ca^{2+}-free solution did contract in response to hyperosmolar K^+-rich solutions. This observation is consistent with a previous report by Granger et al. (1986) showing that the K^+-induced effect in the rat uterus is markedly reduced, but not abolished, after 1 or 2 h incubation in a Ca^{2+}-free solution with or without EGTA. The response to hyperosmotic K^+, which remained in Ca^{2+}-free solution, could occur as a consequence of cell volume decrease, due to hyperosmotic stress (Nielsen-Kudsk et al., 1992; Takeda et al., 1993). This hypothesis is unlikely since hyperosmotic solutions of sucrose or mannitol induced much smaller contractions, which were unaffected by removal of external Ca^{2+} or nifedipine. Moreover, the contractions induced by sucrose in both depolarized (K^+ 60 mM) and non-depolarized Ca^{2+}-free solutions were significantly smaller in size than those evoked by equiosmolar concentrations of K^+. This suggests that hyperosmotic KCl-induced contractions cannot be ascribed to hyperosmolarity per se or to membrane depolarization plus hyperosmolarity and seem to involve activation of additional mechanisms as in other smooth muscles (Low et al., 1994).

It has been reported that, in addition to inducing Ca^{2+} influx, hyperosmotic KCl may stimulate phosphoinositide breakdown (Khac et al., 1992) and mobilize Ca^{2+} from intracellular stores (Somlyo et al., 1991; Munro and Wendt, 1994). The release of internal Ca^{2+} could account for the component of the hyperosmotic KCl-induced contraction remaining in Ca^{2+}-free solution. Indeed, the existence of an internal Ca^{2+} store sensitive to several stimulants (i.e., oxytocin) is clearly established in rat uterine smooth muscle (Anwer and Sanborn, 1989; Kasai et al., 1994). However, the uterine contraction in response to hyperosmotic K^+ in Ca^{2+}-free solution was unaffected by cyclopiazonic acid, by depletion of stored Ca^{2+} with oxytocin, and by

increasing the concentration of EGTA to 10 mM. In addition it could be repeated several times in the same strip without supplying any Ca^{2+}. Taken together, these observations strongly suggest that the responses induced by KCl in Ca^{2+}-free solution do not depend on Ca^{2+} release from internal stores.

Several receptor and non-receptor stimulants have been shown to induce sustained contraction in the rat uterus after prolonged (1 h or more) exposure to a Ca^{2+}-free medium (Sakai et al., 1982; Fukuzaki et al., 1992; Ausina et al., 1996). These responses have been called 'Ca^{2+}-free contractions' because they are produced without any detectable increase in the cytosolic free Ca^{2+} levels (Matsuo et al., 1989; Fukuzaki et al., 1992). A prominent characteristic of these responses is that they are inhibited when the solution is changed to a Ca^{2+}-containing medium (Ishine et al., 1992; Fukuzaki et al., 1992; Miyauchi and Uchida, 1994). Hyperosmolar K^+-rich solutions may cause contraction by activation of mechanisms similar to those involved in 'Ca^{2+}-free contractions'. If such were the case, the Ca^{2+} reversal phenomenon might explain the smaller responses to KCl in Ca^{2+} (0.54 mM)- and nifedipine (1 μM)-containing solution, in comparison to responses obtained in Ca^{2+}-free solution containing 3 or 10 mM EGTA.

In the present experiments, neither W-7 (a Ca^{2+}/calmodulin antagonist, Tanaka et al., 1982) nor KN-62 (a competitive inhibitor of the calmodulin binding of Ca^{2+}/calmodulin kinase II, Tokumitsu et al., 1990) inhibited the contraction induced by hyperosmotic K^+ in Ca^{2+}-free solution. These findings suggest that this contraction does not involve activation of Ca^{2+}/calmodulin dependent mechanisms. Alternatively, the K^+-induced contraction may depend on an increase in the sensitivity of the contractile machinery to Ca^{2+}. It has been suggested that tyrosine kinases may contribute to the mechanism of Ca^{2+} sensitization (Somlyo and Somlyo, 1994). However, the tyrosine kinase inhibitor genistein did not affect the contraction induced by hyperosmotic KCl, suggesting that genistein-sensitive protein tyrosine kinases do not play a major role in mediating this contractile response. Another important kinase thought to be involved in the Ca^{2+}-sensitizing effects of agonists in smooth muscle is protein kinase C (Morgan et al., 1992; Karibe et al., 1991). Low et al. (1994) found that the Ca^{2+}-free contraction induced by hyperosmotic KCl in the rat and guinea-pig aorta involved protein kinase C activation. In our experiments, calphostin C was only a weak inhibitor of the contraction induced by hyperosmotic K^+, suggesting that protein kinase C plays a minor role, if any, in sustaining the KCl-induced contraction in the rat uterus. In contrast, calphostin C did reduce the contractile response evoked by sucrose in similar experimental conditions. The response to sucrose was also depressed in the presence of H-7, staurosporine and genistein. It thus appears that protein kinase C and protein tyrosine kinase(s) are activated in the rat uterus as a consequence of cell shrinkage, due to hyperosmolarity, as previously observed in other types of cell (Takeda et al., 1993). This finding, together with the observation that the sucrose-induced response disappeared on a second or third application, provides further evidence for the hyperosmotically added KCl-induced contraction being mainly mediated by mechanisms different from those activated by hyperosmotic stress, to which uterine cells seem to be only slightly responsive.

In contrast to calphostin C, H-7 and staurosporine inhibited partially (100 μM H-7) or almost abolished (300 nM staurosporine) the hyperosmotic KCl-induced contraction. Although these agents have been widely used as protein kinase C inhibitors, it is well known that they are poorly selective. Moreover, at the concentrations used in the present study they may inhibit other kinases. Both H-7 and staurosporine bind to the ATP binding site on protein kinase C (Hidaka et al., 1984), a region with a high degree of sequence homology in most kinases (Edelman et al., 1987). Staurosporine has been reported to inhibit Ca^{2+}/calmodulin-dependent and independent activities of Ca^{2+}/calmodulin kinase II (Yanagihara et al., 1991) and also inhibit protein tyrosine kinases (Nakano et al., 1987). However, these kinases cannot mediate the inhibitory effects shown by staurosporine in the present study since, as mentioned above, KN-62 and genistein were ineffective as inhibitors of the hyperosmotic KCl-induced contraction. Myosin light chain kinase is another enzyme with an ATP binding site similar to protein kinase C (Edelman et al., 1987). The inhibition of this or of another, at presently unidentified, kinase may explain the inhibitory effects of staurosporine on the KCl-induced response.

In conclusion, the present data show that the rat uterus is only slightly responsive to cell volume decrease caused by hyperosmotic stress. Hyperosmotic K^+ is able to induce a contraction which is independent of both extracellular and intracellular sources of Ca^{2+} and seems also to be mainly independent of membrane depolarization and/or hyperosmolarity per se. The contraction does not seem to be mediated by protein kinase C, Ca^{2+}/calmodulin-dependent kinases or protein tyrosine kinases but involves activation of other(s), at the present unknown, staurosporine-sensitive protein kinase(s). We have previously found that incubation of the rat uterus in a K^+-free solution inhibited the Ca^{2+}-free, sustained contractions induced by various agonists (Ausina et al., 1996). These and the present findings suggest that K^+ ions could play a more important role in controlling myometrial contractility than that derived from simple modification of the membrane potential.

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