

## The Inhibitory Effects of Frusemide on $\text{Ca}^{2+}$ Influx Pathways Associated with Oxytocin-induced Contractions of Rat Myometrium

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**Abstract.** Contractile responses induced by 25  $\mu\text{mol/l}$  oxytocin in myometrial strips isolated from the uterus of estradiol-dominated rats comprised both phasic and tonic components. In a  $\text{Ca}^{2+}$ -free medium (containing 0.1 mmol/l EGTA and no added  $\text{Ca}^{2+}$ ), the oxytocin-induced contractions seemed to be associated with  $\text{Ca}^{2+}$  release from intracellular stores. Frusemide, known to lower the cAMP level in the rat myometrium, did not affect the responses due to  $\text{Ca}^{2+}$  release but inhibited those mediated through an acceleration of the  $\text{Ca}^{2+}$  influx. The permanent presence of frusemide (1.5 mmol/l) in the  $\text{CaCl}_2$ -containing medium influenced the oxytocin-induced responses in the same manner as did omission of  $\text{Ca}^{2+}$  from the medium. The frusemide-sensitive component of the responses to oxytocin was superimposed on a persistent contraction caused by KCl depolarization, suggesting that frusemide completely inhibited the oxytocin-induced  $\text{Ca}^{2+}$  influx. At the same time, frusemide moderately (by only  $34 \pm 7\%$ ) decreased the amplitude of the KCl-induced contracture. This decrease varied with the frusemide concentration, and could be partly prevented by addition of dibutyryl-cAMP; i.e. probably, it was mediated by an inhibition of voltage-gated  $\text{Ca}^{2+}$  influx due to a decrease in the intracellular cAMP level. The data presented seem to suggest that in the rat myometrium exposed to oxytocin (25  $\mu\text{mol/l}$ ) both voltage-gated and receptor-operated  $\text{Ca}^{2+}$  entries are regulated by cAMP-dependent protein kinases.

**Key words:** Oxytocin — Frusemide — Rat myometrium

### Introduction

Myometrial contractions are obviously governed through elevation in intracellular free  $\text{Ca}^{2+}$  due to its release from intracellular stores and/or its influx across the plasma membrane through  $\text{Ca}^{2+}$  channels. At low ( $\sim\text{nmol/l}$ ) concentrations of oxytocin (OT) regular phasic myometrial contractions are activated by a rise

in the intracellular free  $\text{Ca}^{2+}$  which is likely to be predominantly derived from an influx across the plasma membrane (Mironneau 1976; Edwards et al. 1986). Two types of  $\text{Ca}^{2+}$  channels have been identified in smooth muscle plasma membranes: voltage-gated and receptor-operated (Edwards et al. 1986), but it appears that the great bulk of intracellular  $\text{Ca}^{2+}$  elevation during oxytocin-induced phasic contractions in the rat myometrium comes through the voltage-gated  $\text{Ca}^{2+}$  channels (Mironneau 1976; Edwards et al. 1986). However, the triggering role of the receptor-operated  $\text{Ca}^{2+}$  channels in the development of responses cannot be excluded (Bolton 1979). At higher oxytocin concentrations, a sustained increase in basal tone can be recorded in addition to phasic contractions. It has been suggested that  $\text{Ca}^{2+}$  required for increasing the basal tone is derived from internal stores (Flint et al. 1986). In the myometrium isolated from ovariectomized sheep (Flint et al. 1986) and pregnant guinea-pigs (Marc et al. 1986) oxytocin at micromolar concentrations was found to elevate intracellular inositol triphosphate ( $\text{IP}_3$ ) in a dose-dependent manner. A similar dependence was obtained for the myometrium of pregnant rats, with the  $\text{IP}_3$  formation in response to OT at concentrations of the same order of magnitude being followed by  $\text{Ca}^{2+}$  release from the intracellular stores (Ver et al. 1989). An analysis of the above facts suggests that two different mechanisms are responsible for the development of phasic and tonic components of OT-induced contractile responses.

Recently, it has been shown that frusemide, a widely used diuretic, is capable of inhibiting phasic contractions in the myometrium of estradiol-treated rats in response to low (nanomolar) concentrations of oxytocin (Mozhayeva et al. 1994). This effect may be associated with an unusual effect of frusemide in this tissue: stimulation of cAMP- phosphodiesterase (cAMP-PDE) activity and reduction of the intracellular cAMP level (Bagrov et al. 1993). The precise mechanism of the action of frusemide remains to be identified.

The present experiments were designed to test possible effects of frusemide on each of the pathways responsible for the elevation in intracellular  $\text{Ca}^{2+}$ . This was done using  $\text{K}^+$  depolarization to activate exclusively the voltage-dependent channels, and by high concentrations of oxytocin ( $\sim 25 \mu\text{mol/l}$ ) to activate all the three major pathways involved in the elevation in intracellular  $\text{Ca}^{2+}$ : receptor-operated ion channels, voltage-gated ones, and  $\text{IP}_3$ -dependent mobilization from intracellular stores.

## Materials and Methods

The method used to measure force production in rat myometrial strips has been described in detail previously (Mozhayeva et al. 1994). Briefly, female Wistar rats were injected with estradiol dipropionate, 48 and 24 h before the experiment. The rats were killed by a blow to the head, and the uterus horns were isolated. Longitudinal strips ( $1 \times 10 \text{ mm}$ )

were prepared and mounted vertically in a 3 ml jacketed- bath at 37°C. The preparation was superfused with bathing solution at the rate of 1 ml/min. The standard bathing solution contained (mmol/l): NaCl 154; KCl 11.3; CaCl<sub>2</sub> 0.2; NaHCO<sub>3</sub> 5.9; glucose 2.8; HEPES 10; pH 7.4 (37°C). Modified solution with an elevated K<sup>+</sup> contained (mmol/l): KCl 140; MgCl<sub>2</sub> 1.2; CaCl<sub>2</sub> 2; glucose 11; HEPES 10; pH 7.4 (37°C). Two solutions with elevated K<sup>+</sup> were used. One contained 0.2 mmol/l CaCl<sub>2</sub>, while the other one contained 2 mmol/l CaCl<sub>2</sub>. For Ca-free solutions, CaCl<sub>2</sub> was omitted from the standard solution while EGTA (final concentration of 0.1 mmol/l) was added. All solutions were bubbled with O<sub>2</sub>.

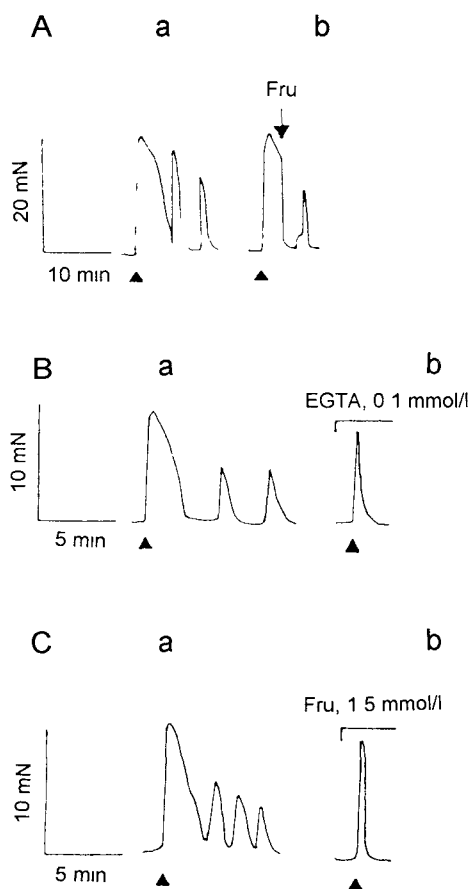
The contractile responses were recorded isometrically and recorded using a pen recorder. Frusemide was added in two ways: either directly into the bath, i.e. transiently, or by addition to the superfusion solution. Oxytocin (25 µmol/l), verapamil (50 µmol/l), and nifedipine (10 µmol/l) were added to the bath only. Prior to each experiment the responsiveness of each preparation to oxytocin was checked. If repeated exposures to oxytocin did not result in contractions of similar amplitudes, the preparation was discarded.

The following substances were used: oxytocin (Gedeon Richter, Budapest, Hungary), frusemide (Hoechst, Bombay, India), dibutyryl-cAMP, EGTA, HEPES (Sigma, St. Louis, USA), verapamil (isoptin) (LEK, Lyublana, Yugoslavia).

## Results

Figure 1 shows typical changes in force production in myometrial strips in response to single brief applications of high doses of oxytocin (25 µmol/l). In each experiment illustrated, the control response (panels *a*), recorded in a bathing solution containing 0.2 mmol/l Ca<sup>2+</sup>, consisted of a large transient contracture which was followed, as oxytocin was washed from the bath, by several phasic contractions. During the contracture, the Ca<sup>2+</sup> required for force production is derived from internal stores and from the external medium. Omission of Ca<sup>2+</sup> from the bathing fluid resulted in a decrease of both the amplitude and the duration of the total OT-induced responses (Fig. 1*Bb*). Thus, only a small part of the total OT-induced contractions was conditioned by Ca<sup>2+</sup> released from IP<sub>3</sub>-sensitive stores, while the rest was associated with Ca<sup>2+</sup> entry. When frusemide (1.5 mmol/l) was added to the bathing solution during the elevated phase of the contracture, the myometrium rapidly relaxed to pre-stimulation levels (Fig. 1*Ab*). This inhibitory action of frusemide was observed in all experiments (*n* = 8). The addition of frusemide prior to stimulation with oxytocin (Fig. 1*Cb*) dramatically affected the time course of the contraction, which was similar to OT-induced response in Ca<sup>2+</sup>-free media. Taken together, these observations suggest that the inhibitory effect of frusemide is a consequence of an inhibition of Ca<sup>2+</sup> influx rather than of the release of Ca<sup>2+</sup> from the intracellular stores. It should be noted that the frusemide action could not be explained by its direct influence on either the contractile apparatus or mechanism(s) lowering Ca<sup>2+</sup> concentration in the myoplasm.

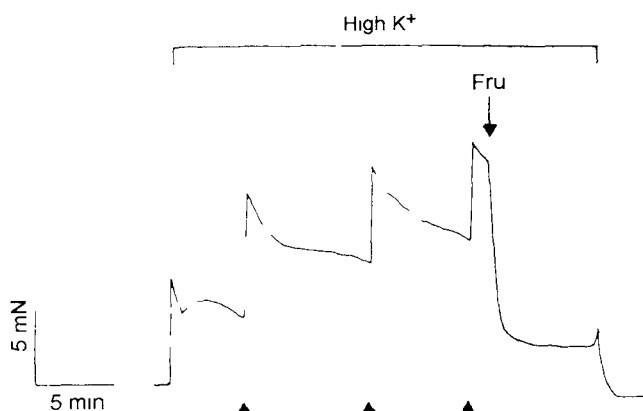
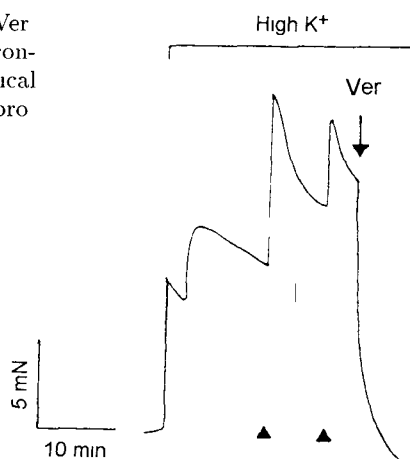
In order to explore the actions of frusemide on the Ca<sup>2+</sup> influx pathways, fur-



**Figure 1.** Effect of  $\text{Ca}^{2+}$  removal from the external medium and of frusemide (1.5 mmol/l) application on oxytocin (25  $\mu\text{mol/l}$ )-induced myometrial contractions. Fragments of continuous recordings are shown. The calibration bars show force (mN) and time (min). Where indicated by triangles, oxytocin was added to the bath to a final concentration of 25  $\mu\text{mol/l}$ . Additions to the superfusion solution are shown by the horizontal bar. Panels *a* in A – C illustrate the oxytocin-induced myometrial contractions in standard solution (0.2 mmol/l  $\text{Ca}^{2+}$ ); *Ab* – Effect of frusemide (Fru) added to the bath medium on the decline of the contractile responses to oxytocin (representative recording of 8 experiments); *Bb* Influence of the omission of  $\text{Ca}^{2+}$  from the medium on the oxytocin-induced response, *Cb* – Effect of frusemide added to the standard medium prior to oxytocin application (representative recording of 6 experiments).

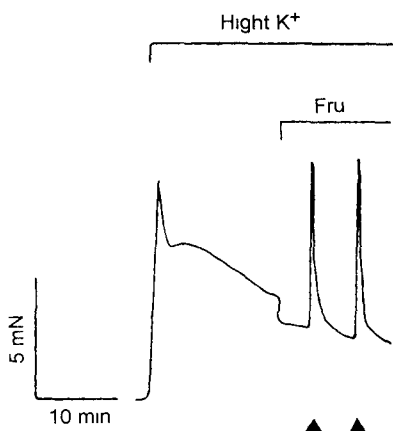
ther experiments were done using high  $\text{K}^{+}$  solutions to elicit contractures. Under these conditions, the  $\text{Ca}^{2+}$  responsible for the contracture is derived from the external medium and very probably enters the cell via voltage-gated ion channels. Examples of  $\text{K}^{+}$ -contractures are shown in Figures 2–5. In each case, the contracture consists of a rapid increase in force which reaches a maximum in a few seconds. Thereafter, the force slowly declines over the following 30–40 minutes. Brief applications of oxytocin (25  $\mu\text{mol/l}$ ) to myometrial strips already in contracture due to exposure to high  $\text{K}^{+}$  medium were found to result in a further increase in force production (Fig. 2). These preparations did not show any phasic activity as the oxytocin was washed out of the bath (see also Fig. 3), as contrasted to the standard conditions (Fig. 1, panels *a*). The shape and the amplitude of OT-induced responses were the same as in control (Fig. 1 *Aa*), with the responses

**Figure 2.** Effect of verapamil (Ver 50  $\mu\text{mol/l}$ ) on oxytocin induced contractions in high  $\text{K}^+$  medium. Typical recording, with the effect being reproducible in all 4 experiments



**Figure 3.** Influence of frusemide (Fru 1.5 mmol/l) on oxytocin-induced contractions in high  $\text{K}^+$  medium. Typical recording (of 4) with the effect being reproducible in all 4 experiments

being superimposed on the  $\text{K}^+$  contractions. Owing to this it can be suggested that the  $\text{Ca}^{2+}$  mediating the OT-induced contractions in these circumstances was not only derived from  $\text{Ca}^{2+}$  release but also from  $\text{Ca}^{2+}$  entry. Addition of the voltage-dependent  $\text{Ca}^{2+}$  channel blocker verapamil (Fig. 2) or nifedipine (data not

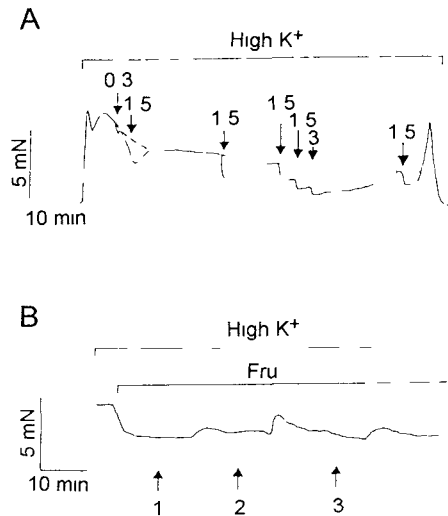


**Figure 4.** Oxytocin-induced contractions in high  $K^+$  medium containing frusemide (Fru, 1.5 mmol/l). Typical recording (of 3), with the effect being reproducible in all 3 experiments.

shown) was found to result in both the inhibition of OT-induced responses associated with the  $Ca^{2+}$  entry and the relaxation of the  $K^+$ -contracture maintained with the involvement of the voltage-gated channels. The addition of frusemide was also observed to cause myometrial relaxation (Fig. 3). Small residual component of  $K^+$ -contractures was recorded nevertheless.

When a  $K^+$ -precontracted myometrial strip was exposed to frusemide, there was a small fall in force production. Subsequent brief applications of oxytocin activated further contractures (Fig. 4). These contractures were brief and similar to those seen in solutions containing usual  $K^+$  concentration in the presence of frusemide (Fig. 1*Cb*) and those in a nominally  $Ca^{2+}$ -free solution (Fig. 1*Bb*). This suggests that the  $Ca^{2+}$  responsible for these brief contractions was derived from internal stores.

Figure 5*A* shows that, in a single experiment, brief pulses of frusemide at concentrations from 0.3 to 3 mmol/l can repeatedly cause relaxation in  $K^+$ -induced contracture. Even at high frusemide concentrations, the relaxation was never complete (max.  $34 \pm 7\%$ ,  $n = 45$ ). Previous work on the rat myometrium has suggested that the inhibitory actions of frusemide are associated with the activation of cAMP-PDE and a subsequent fall in intracellular cAMP (Bagrov et al. 1993; Mozhayeva et al. 1994). Evidence to support this idea is shown in Fig. 5*B*. Contracture was induced in myometrial strips by using bathing solution with an elevated  $K^+$  concentration, and relaxation was induced by subsequent exposure to frusemide. When dibutyryl- cAMP was added to the superfusion solution, transient increases in force were recorded, supporting the idea that the relaxation is a consequence of a fall in intracellular cAMP. In contrast, as shown in our earlier work (Mozhayeva



**Figure 5.** Effects of frusemide and dibutyryl-cAMP on the amplitude of the  $K^+$  contracture. *A* – The application of frusemide to the medium induces reversible reduction of  $K^+$  contracture amplitude. The addition of frusemide at various concentrations to the bath is shown by vertical arrows; *B* – The application of dibutyryl-cAMP induces partial restoration of the decrease of the  $K^+$  contracture tension evoked by the presence of frusemide (Fru, 1.5 mmol/l) in the media. The arrows mark the dibutyryl-cAMP addition at various concentrations (in  $\mu\text{mol/l}$ ). Typical recording (of 4), with the effect being reproducible in all 4 experiments.

et al. 1994), in control experiments as little as 1  $\mu\text{mol/l}$  dibutyryl-cAMP evoked the relaxing effect.

## Discussion

Data presented in this paper show that frusemide can induce relaxation in precontracted myometrial strips isolated from estradiol-dominated rats. Relaxation was observed in tissues contracted either by pretreatment with high doses of oxytocin (25  $\mu\text{mol/l}$ ) or due to  $K^+$ -depolarization.

The contractions induced by oxytocin can be activated both in the presence and in the nominal absence of  $\text{Ca}^{2+}$  in the bathing solution. These observations confirm that the  $\text{Ca}^{2+}$  required for the contractions is derived from two sources: intracellular stores and external medium. The mobilization of  $\text{Ca}^{2+}$  from internal stores is linked to the formation of  $\text{IP}_3$  (Flint et al. 1986; Marc et al. 1986) and a subsequent discharge of the  $\text{Ca}^{2+}$  stores (Ver et al. 1989). Frusemide had no effect on the oxytocin-induced contractions in a nominally  $\text{Ca}^{2+}$ -free solution, suggesting

that the relaxation action of frusemide was not associated with formation of  $IP_3$ , mobilization and extrusion of intracellular  $Ca^{2+}$  or activation of the contractile apparatus.

It may therefore be concluded that the actions of frusemide are associated with mechanisms responsible for the influx of  $Ca^{2+}$  across the cell membrane. Exposure of the preparations to solutions containing high  $K^+$  resulted in contracture which is associated with the participation of voltage-gated  $Ca^{2+}$  channels. Since the oxytocin-induced responses were superimposed on the  $K^+$ -induced contractions (Figs. 2 and 3) and occurred in depolarized preparations, it can be suggested that  $Ca^{2+}$  entry may be a result of an operation of at least two mechanisms: (1) OT receptor-operated channels, and (2) store depletion dependent  $Ca^{2+}$  influx (Putney 1992).

Frusemide is widely known to inhibit Na-K-Cl co-transport (see Geck and Heinz 1986) and  $Cl^-$  channels (see Greger 1985). Nonetheless, when investigating its effects in the rat uterus, we (Bagrov et al. 1993; Mozhayeva et al. 1994) could draw the conclusion that our findings may only be accounted for by the activatory action of frusemide on cAMP-PDE. The results of the present work provide an additional support for this assumption. Indeed, had the frusemide influence on OT-induced contractile responses been due to inhibition of the ion transport, its effects would have differed depending on the electrolyte content of the bathing fluid (normal and high potassium solution).

Earlier we have found that the inhibitory effect of frusemide could be reversed by elevating the intracellular cAMP level as affected by theophylline (Bagrov et al. 1993) or dibutyryl-cAMP (Mozhayeva et al. 1994). Our present results show that frusemide partly affects the KCl-induced contracture and this relaxing effect can be reversed either by its removal (Fig. 5A) or by addition of a certain amount of dibutyryl-cAMP (Fig. 5B). There is an evidence that receptor-operated  $Ca^{2+}$  channels in bovine trachea smooth muscle can be regulated by cAMP, with an increase in cAMP resulting in an acceleration of  $Ca^{2+}$  influx (Takuwa et al. 1988). So, vasopressin-induced  $Ca^{2+}$  influx in vascular smooth muscle cells increased as cAMP concentration in the bathing medium was raised (Lincoln et al. 1990). More widely known is the cAMP-dependence of voltage-operated  $Ca^{2+}$  channels of L-type, first described by Reuter et al. (1982) for cardiomyocytes and since then discovered in various tissues (see Ichida et al. 1984). So, it is possible that a mechanism involving cAMP is responsible for the effect of frusemide on the voltage-gated and OT-induced  $Ca^{2+}$  influx.

It is noteworthy that the frusemide influence on the KCl-induced contracture was rather modest as compared to its effect on the oxytocin-induced responses. This distinction might reflect a difference in the sensitivity of receptor-operated and voltage-gated  $Ca^{2+}$  channels to the cAMP level. However, on smooth muscles it has been shown that KCl-depolarization lowers the sensitivity of myosin light chain



phosphorylation towards  $\text{Ca}^{2+}$  and that cAMP-dependent mechanisms involved into relaxation of KCl-induced contracture and of agonist-induced contractions are distinct (McDaniel et al. 1991; Tang et al. 1992). Thus, at this point it is not possible to compare the cAMP-sensitivity of receptor-operated and voltage-gated  $\text{Ca}^{2+}$  channels in the rat myometrium. This problem requires further investigation.

A comparison of the present results with those obtained under the same conditions but at low concentrations of oxytocin (Mozhayeva et al. 1994) allows to assume that there are two types of oxytocin receptors on the rat myometrium plasma membrane. Such an assumption is supported by the fact that oxytocin is efficient over a concentration range as wide as three orders, so that the responses to the hormone at high concentrations are mediated through  $\text{Ca}^{2+}$  release from  $\text{IP}_3$ -sensitive stores together with its receptor-operated (or store depletion-dependent) influx, while at low concentrations oxytocin induces basal oscillations associated with the operation of voltage-gated  $\text{Ca}^{2+}$  channels but not with the  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release.

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