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Role of Capsaicin-Sensitive Nerve Fibers in Uterine Contractility in the Rat[1]

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ABSTRACT

The possible participation of capsaicin-sensitive sensory nerves in the modulation of neurogenic contractions was studied in nonpregnant and term pregnant rat uteri. Neurogenic contractions were elicited by electric field stimulation (40 V, 1–70 Hz, 0.6 msec) in intact uteri and uteri that were previously exposed to capsaicin in vitro. In capsaicin pretreated preparations obtained both from nonpregnant and term pregnant rats, a dose-dependent increase in the amplitude of uterine contractions was detected. Prior systemic treatment of the rats with capsaicin (130 mg/kg, s.c.) abolished the effect of in vitro capsaicin administration on the amplitude of neurogenic contractions. Use of a specific antagonist of calcitonin gene-related peptide revealed that depletion of this peptide, which normally elicits uterine smooth muscle relaxation, may be responsible for the increased responsiveness of the uterus to low-frequency stimulation. Experiments on the localization of calcitonin gene-related peptide in uterine tissue specimens exposed to capsaicin revealed dose-dependent depletion of calcitonin-gene related peptide-immunoreactive nerves innervating blood vessels and the myometrium. The findings indicate that capsaicin-sensitive afferent nerves, by the release of sensory neuropeptides, significantly contribute to the modulation of uterine contractility both in nonpregnant and term pregnant rats. It is suggested that uterine sensory nerve activation may be part of a trigger mechanism leading to preterm contractions evoked by, for example, inflammation.

female reproductive tract, neuropeptides, pregnancy, uterus

INTRODUCTION

Uterine and cervical contractility during pregnancy and at term are subjected to neuronal regulation involving autonomic and sensory nerves. The changes in adrenergic receptor density associated with an ongoing degeneration process in the course of pregnancy and their role in the modulation of uterine contractility is well established [1–5]. In most visceral organs, stimulation of sensory nerves may evoke significant alterations in smooth muscle contractile activity [6, 7]. Anatomical, neurochemical and pharmacological studies revealed that this may be attributed to the effects of neuropeptides released from stimulated sensory nerve terminals [8–10]. This particular local regulatory or sensory efferent function is a characteristic trait of primary afferent neurons that are specifically sensitive to capsaicin [11–13] and express the capsaicin/vanilloid VR1 receptor [14, 15]. Capsaicin-sensitive afferent nerves have been demonstrated by immunohistochemistry in the uterus of several species including the rat. These nerves contain calcitonin gene-related peptide (CGRP), substance P (SP), galanin (GAL), and neurokinin A (NKA) alone or colocalized [16–18]. During pregnancy, certain types of sensory nerve fibers in the rat uterus may undergo degeneration. This neuroplastic change of the pregnant uterus is a known, yet not fully described or understood phenomenon. Some immunocytochemical studies have indicated that all the neuropeptide immunoreactivities has vanished in full-term pregnant guinea pigs [19]. However, there is some controversy as to the nature and extent of these changes. Some authors reported the disappearance of myometrial and perivascular vasoactive intestinal polypeptide (VIP)-containing fibers at the end of pregnancy [20], whereas others provided evidence for the presence of SP- and CGRP-containing sensory nerves at term [21]. It has been suggested that uterine nerves contribute to the implantation processes and the maintenance of pregnancy, and they may play a role in the increase of uterine contractility and in cervical ripening around term [22, 23].

In vivo and in vitro studies showed that SP, NKA, and GAL elicit contraction [16, 17, 24], whereas CGRP and VIP [25, 26] cause relaxation of uterine smooth muscle in nonpregnant uteri. Although the effects of these peptides on uterine smooth muscle activity is well established, the possible participation of capsaicin-sensitive afferent nerves in the modulation of uterine contractility has not been dealt with in detail. Therefore, the aim of the present study was to elucidate the possible functional significance of this particular class of sensory nerves in the modulation of uterine contractility in nonpregnant and in term (22 day) pregnant rats. Using electric field stimulation, we elicited selective nerve-induced contractions and examined the effect of selective chemodenervation by capsaicin on the contractile responses of uterine preparations of nonpregnant and term pregnant rats. We performed experiments on the localization of CGRP by immunohistochemistry in uterine tissue specimens exposed to capsaicin to demonstrate the possible depletion of CGRP-immunoreactive nerves innervating blood vessels and the myometrium.

MATERIALS AND METHODS

Animals

Animal investigations were carried out with the approval of the Ethical Committee for Animal Research, University of Szeged (Registration no. I-74-8/2002).

Sexually mature female Sprague-Dawley rats (body mass: 180–240 g, 60–80 days old) were used in the experiments. Mating with males was carried out in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of conception was considered to be the first day of pregnancy; animals used in the experiments were 22-day (term) pregnant rats. Nonpregnant females

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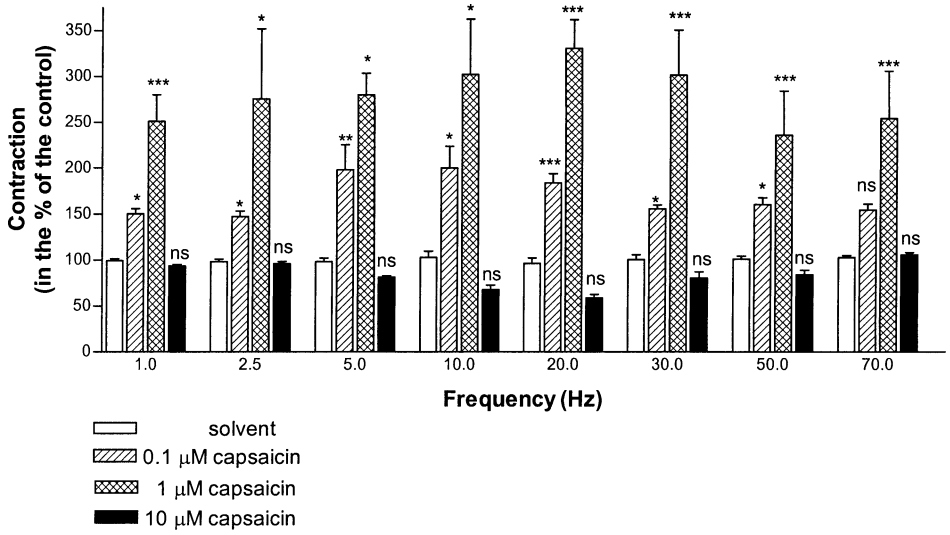

FIG. 1. The effects of in vitro capsaicin treatment on the contraction responses evoked by electric field stimulation in isolated uterine rings of nonpregnant rats. The amplitudes of the contractions after a 20-min incubation period with capsaicin are expressed as percentages of the control contractions elicited at the same frequency. Each bar represents mean ± SEM, n = 6. Data were statistically analyzed at each frequency value independently. The asterisks indicate the level of significant difference from the solvent treated preparations (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns means $P > 0.05$, thus no significant difference).

were cyclic; virgin rats held in separate cages from males since the third week after birth. At the time of the experiments, they were in the diestrus phase. The animals were housed in temperature (20–22°C) and humidity controlled (40%) and light regulated (12L:12D) rooms with water and food intake ad libitum.

Uterus preparation and electric field stimulation parameters

The rats were killed by cervical dislocation at 1000 h, the uteri were removed and trimmed of fat, and the fetoplacental units were removed from the pregnant animals. The uteri were immediately placed in an organ bath (de Jongh solution containing in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 Na_2HPO_4, 6 glucose; pH 7.4), perfused with 95% oxygen and 5% carbon dioxide. Temperature was maintained at 37°C. Rings 1 cm long were sliced from the middle part of each horn, including implantation sites in case of pregnant animals, and mounted vertically between two platinum electrodes in 10 ml of the above-mentioned organ bath under the same conditions. After mounting, the uterine rings were equilibrated for 90 min before the experiment. The initial tension was set at 1.5 g. After the incubation period, selective nerve stimulation was elicited by a digital, programmable stimulator (ST-02, Experimetria Ltd., London, UK). The square pulse duration was set at 0.6 msec and the supramaximal voltage at 40 V. Rings were stimulated by 30-sec trains of square pulses with the following frequencies, in increasing order: 1, 2.5, 5, 10, 20, 30, 50, and 70 Hz. Each 30-sec stimulation was followed by a 2-min recovery period, and then a higher frequency was applied. The first stimulation series was regarded as a control. After the first series of stimulation, the tissue samples were washed thoroughly and then incubated for 20 min with 0.1–10 μM capsaicin (dissolved in physiological saline containing 6% Tween 80 and 8% ethanol; purchased from Fluka Chemie AG, Budapest, Hungary). After this, the uterine rings were washed out and stimulated again with the same series of frequencies (1–70 Hz). The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd.) and recorded and analyzed by ISOSYS Data Acquisition System (Experimetria Ltd.). The alteration of the amplitude caused by capsaicin administration was expressed as a percentage of the control contraction.

In some experiments on nonpregnant rat uteri, no capsaicin was added to the organ bath during the 20-min incubation period, and the second series of stimulation was performed in the presence of the CGRP1 receptor subtype antagonist, hCGRP(8-37) (dissolved in distilled water (Sigma Aldrich Ltd., Budapest, Hungary). The organ bath contained 1–10 μM of hCGRP(8-37) (final concentration), and stimulation was performed as described above (same series of frequencies: 1–70 Hz).

In vivo capsaicin treatment

Under ether anesthesia, capsaicin was administered to adult nonpregnant female rats in three s.c. injections (10, 20, and 100 mg/kg capsaicin, respectively, dissolved in physiological saline containing 6% Tween 80 and 8% ethanol on days 1, 2, and 4, respectively). The experiments were performed 4 days after the last dose.

Immunohistochemistry

Uterine rings were placed in an organ bath containing de Jongh solution perfused with 95% oxygen and 5% carbon dioxide, at 37°C. Thirty minutes later, capsaicin was added at a final concentration of 1–10 μM. Solvent-treated specimens served as controls. After an additional period of 20 min, this solution was replaced with fresh physiological saline. Ten minutes later, tissue specimens were placed in Zamboni fixative for 2 h at 4°C. The samples were washed in phosphate buffer (0.1 M, pH 7.4) and processed for the demonstration of CGRP immunoreactivity (IR) using an indirect immunofluorescence technique. Briefly, cryostat sections 15 μm in thickness were cut and incubated with a rabbit polyclonal CGRP antibody (Sigma Chemical Co., St. Louis, MO; 1:500) overnight. After repeated washing with PBS, the sections were incubated with a secondary antibody (goat anti-rabbit IgG conjugated to Cy3) for 1 h at room temperature. After washing in buffer, tissue sections were covered with Cytifluor (Amersham, Arlington Heights, IL) and examined under a DMLB fluorescence microscope (Leica, Wetzlar, Germany).

Statistical Analyses

All experiments were carried out on at least six animals and values are given as mean ± SEM. Bartlett tests revealed the homogeneity of variances. One-way ANOVA with Newman-Keuls test was used to compare the amplitudes of given contractile responses at each frequency independently. The recorded data were statistically analyzed with the Prism 2.01 (Graph Pad Software Inc., San Diego, CA) computer program.

RESULTS

Figure 1 illustrates the effects of 0.1–10 μM capsaicin administration in vitro on the amplitudes of the neurally evoked contractions of the isolated nonpregnant rat uteri. At the lowest dose (0.1 μM), the amplitudes increased significantly, compared with the solvent-treated control when the uterine rings were stimulated at 1–50 Hz. After incubation with 1 μM capsaicin, the uteri gave significantly greater responses to 1- to 70-Hz frequency stimulation than those of the samples that were treated with solvent only. Incubation with 10 μM capsaicin did not cause any alteration of contraction responses. The greatest contractions were detected after incubation with 1 μM capsaicin.

To exclude the possibility of a direct action of capsaicin on the uterine smooth muscle, a group of nonpregnant rats were pretreated with 130 mg/kg capsaicin, and 4 days after the last day of administration, the same experiment was carried out: the effect of 1 μM capsaicin on the uterine contractions in vitro was tested. Enhancement of the amplitude of the uterine contractions after incubation with 1 μM capsaicin was not observed when rats were pretreated

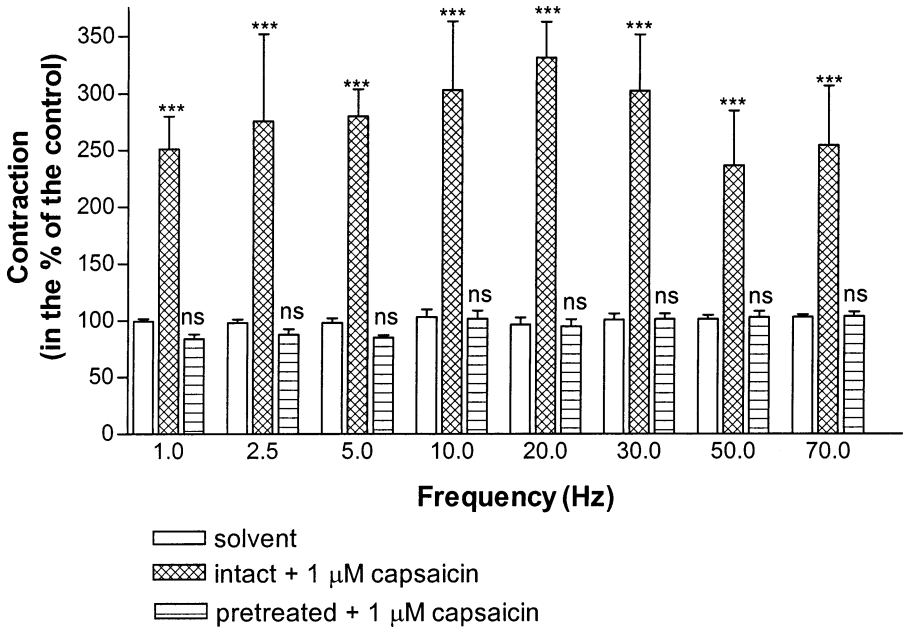

FIG. 2. The effects of in vitro 1 μM capsaicin treatment on the contraction responses evoked by electric field stimulation in isolated uterine rings of intact and in vivo capsaicin-pretreated (130 mg/kg, s.c.) nonpregnant rats. The amplitudes of the contractions after a 20-min incubation period with capsaicin are expressed as percentages of the control contractions, elicited at the same frequency. Each bar represents mean ± SEM, n = 6. Data were statistically analyzed at each frequency value independently. The asterisks indicate the level of significant difference from the solvent-treated preparations (*** $P < 0.001$; ns means $P > 0.05$, thus no significant difference).

with capsaicin in vivo. The amplitude of the contractions after incubation with 1 μM capsaicin differed significantly in capsaicin-pretreated nonpregnant rat uteri from that in intact nonpregnant rat uteri (Fig. 2).

The effect of the CGRP1 receptor antagonist hCGRP(8-37) was also tested on the neurally evoked uterine contractions (Fig. 3). After the first series of stimulation, the uterine rings were allowed to rest for 20 min and then washed, and hCGRP(8-37) was added to the incubation buffer. The second series of stimulation was performed in the presence of 1 or 10 μM hCGRP(8-37). Stimulated at 1–30 Hz, the amplitude of contractions were significantly higher in the presence of hCGRP(8-37) than in the presence of solvent only. The greatest enhancement of the amplitude was observed when the uterine rings were stimulated with 1 Hz in the presence of 10 μM hCGRP(8-37). In that case the alteration in the amplitude of the contraction response was similar to that observed after the uteri were incubated with 1 μM capsaicin. When uteri were stimulated at higher frequencies, the effect of hCGRP(8-37) on the amplitude of contractions was significantly smaller than that of 1 μM capsaicin.

In agreement with previous findings, immunohistochemistry revealed a dense innervation of uterine blood vessels and smooth muscle in control (solvent-treated) specimens (Fig. 4a). Exposure to capsaicin at concentrations of 0.1–1 μM produced marked depletion of CGRP from both perivascular and myometrial nerve fibers (Fig. 4b). In contrast, capsaicin administered at a concentration of 10 μM produced an almost complete disappearance of all CGRP-IR nerves from the tissue (Fig. 4c). Exposure to capsaicin produced similar effects in the term pregnant rat uterus, although the density of CGRP-IR nerve fibers was markedly reduced, compared with nonpregnant control rats (data not shown).

The effect of capsaicin on nerve-induced contractions was also tested in the term pregnant rat uterus (Fig. 5). Low-frequency stimulation (1–10 Hz) was unable to elicit

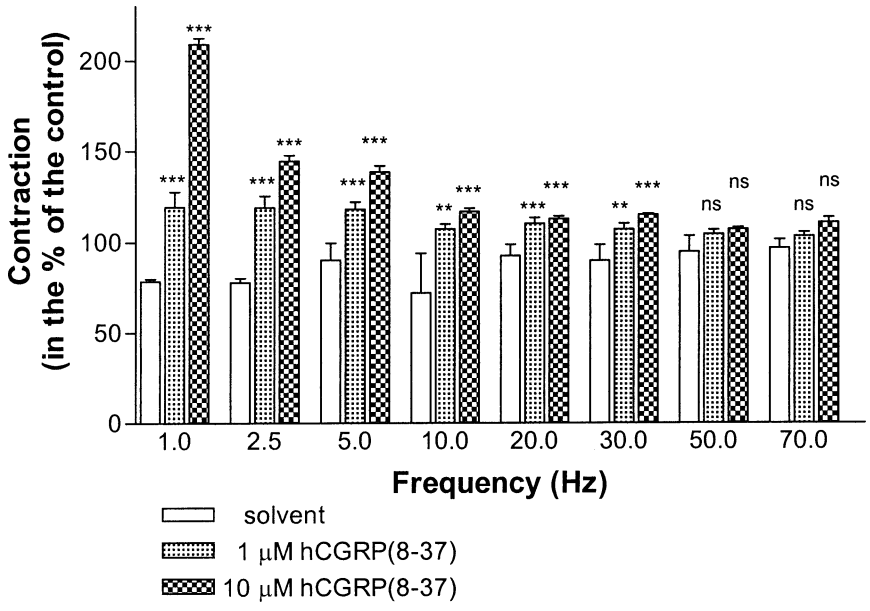

FIG. 3. The effects of hCGRP(8-37) on the contraction responses evoked by electric field stimulation in isolated uterine rings of nonpregnant rats. After a series of control contractions and a 20-min resting period, contractions were evoked by electric field stimulation in the presence of hCGRP(8-37). The amplitudes of the contractions in the presence of hCGRP(8-37) are expressed as percentages of the control contractions, elicited at the same frequency. The effects of in vitro 1 μM capsaicin treatment on the contraction responses evoked by electric field stimulation in isolated uterine rings of intact nonpregnant rats are also shown in the figure. Each bar represents mean ± SEM, n = 6. Data were statistically analyzed at each frequency value independently. The asterisks indicate the level of significant difference from the solvent treated preparations (** $P < 0.01$; *** $P < 0.001$; ns means $P > 0.05$, thus no significant difference).

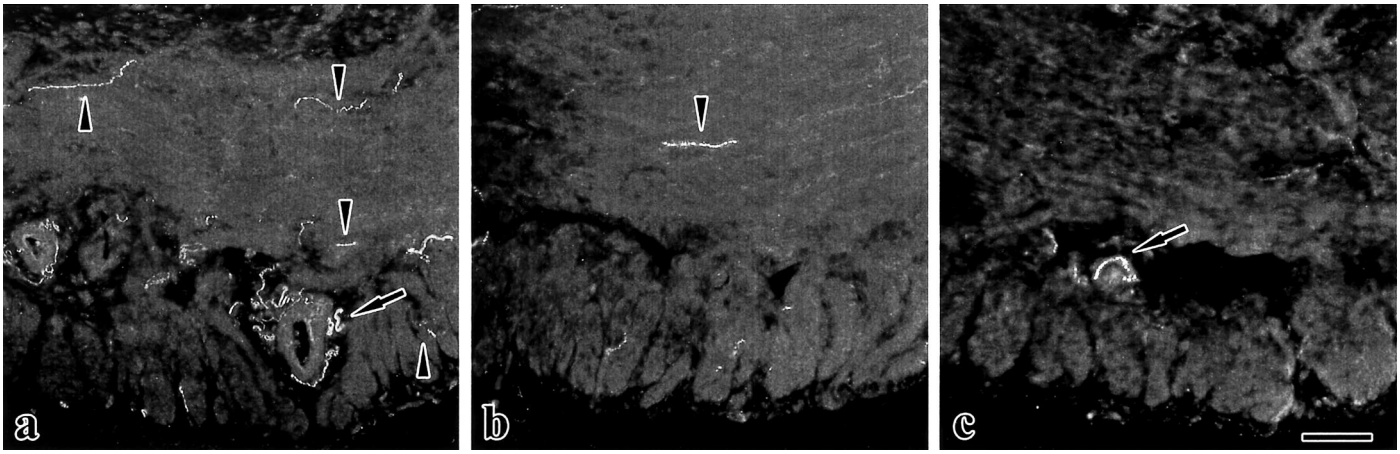

FIG. 4. Immunohistochemical demonstration of CGRP-IR perivascular (arrows) and myometrial (arrowheads) nerve fibers in uterine specimens of the rat. In control samples (**a**) CGRP-IR fibers innervate the blood vessels and the longitudinal and circular smooth muscle layers. Incubation of uterine samples with capsaicin at a concentration of 1μM resulted in a marked depletion of CGRP-IR nerves (**b**). In samples incubated with capsaicin at a concentration of 10 μM only few fragmented degenerating fibers can be seen (**c**). For all figures, bar = 10 μm.

contractions. The effect of 1 μM capsaicin on the amplitude of contractions was quite similar to that in the nonpregnant uteri: a significant increase of the amplitude was observed at each frequency (20–70 Hz), compared with the solvent-treated control. On incubation with the lower dose of capsaicin (0.1 μM), the amplitude of the contractions increased significantly in uteri stimulated at 50–70 Hz. When 20–30 Hz was applied, no significant increase of the amplitude was detected. Similarly as in the nonpregnant uteri, incubation with 10 μM capsaicin did not alter the amplitude of the contraction responses.

DISCUSSION

The possible participation of capsaicin-sensitive sensory nerves in the modulation of neurogenic contractions was studied in nonpregnant and term pregnant rat uteri. Our data demonstrated that capsaicin-sensitive afferents in the nonpregnant rat uterus can be stimulated at a wide range of frequencies. In uterine preparations pretreated with 0.1 or 1 μM capsaicin in vitro, the amplitude of the contractions evoked by stimulation at 1–50 Hz increased dose dependently, which suggested that these parameters were adequate to stimulate capsaicin-sensitive sensory fibers in the rat uterus. These findings are not in complete agreement with some previous ones that have dealt with the excitability of sensory nerves by field stimulation [27–30]. Generally, a higher frequency range (20–70 Hz) is considered to be selective for peptidergic nerves, although neuropeptide release has been measured after stimulation of peptidergic nerves at frequencies as low as 2–10 Hz [31]. However, our studies indicated that low-frequency stimulation (1–10 Hz) also activates capsaicin-sensitive fibers.

The specificity of electric field stimulation to activate nerves instead of smooth muscle directly was ensured by the short pulse width. Transmural nerves can be selectively stimulated by a short pulse duration (<5 msec), whereas visceral smooth muscle requires a longer pulse duration (60–133 msec) for stimulation [32]. On this basis, we applied stimuli 0.6 msec long to elicit neurogenic contractions in the rat uterus. Although the chance of direct smooth muscle stimulation by the electric field was excluded, the possible direct effect of capsaicin on myometrial tissue was also necessary to investigate.

Capsaicin was administered s.c. to a group of nonpregnant rats to evoke an irreversible damage in the sensory nerves, thus to make a negative pharmacological animal model. Four days after the last dose of in vivo capsaicin administration, neurogenic contractions could also be elicited at 1–70 Hz in the isolated uterine preparations from these animals; however, the amplitude of the contractions

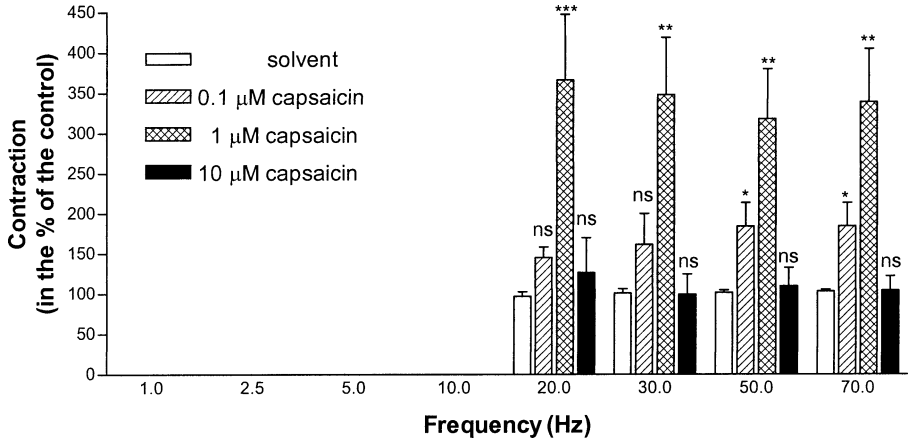

FIG. 5. The effects of in vitro capsaicin treatment on the contraction responses evoked by electric field stimulation in isolated uterine rings of 22-day (term) pregnant rats. The amplitudes of the contractions after a 20-min incubation period with capsaicin are expressed as percentages of the control contractions elicited at the same frequency. The failure to elicit contraction responses at 1–10 Hz electric stimulation is possibly related to the pregnancy-induced uterine nerve degeneration. Each bar represents mean ± SEM, n = 6. Data were statistically analyzed at each frequency value independently. The asterisks indicate the level of significant difference from the solvent-treated preparations (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns means $P > 0.05$, thus no significant difference).

did not change after any dose of in vitro capsaicin treatment. The lack of the effect of in vitro administration of capsaicin was considered to be a consequence of the sensory nerve damage and consecutive neuropeptide depletion [33, 34]. In this way we demonstrated that the contractility-increasing effect of capsaicin is fully dependent on an intact sensory nerve function, and it can be regarded as an indirect action on the myometrium. Furthermore, it is not surprising that neurogenic contractions could be elicited at 1–70 Hz in the uteri of capsaicin-treated animals because the contraction responses to stimulation at 1–70 Hz are mediated via adrenergic nerves, which evidently remain intact after capsaicin administration.

The capsaicin-induced alteration of neurally evoked contraction responses points to the contributory role of capsaicin-sensitive sensory nerves to the motor activity of the rat uterus. Although the augmentation of contraction responses was detected after an in vitro incubation with 0.1 or 1 μM capsaicin, we hypothesized that the phenomenon is related to an altered neuropeptide content in the sensory nerves. Although it is not indicated in the figures, here we notice that capsaicin did not cause any change in the basal uterine tone when it was added to the organ bath for a 20-min incubation period. It is proposed that in this experimental design, capsaicin influences the uterine contractility via causing a nonselective release of different neuropeptides from the sensory nerve endings, which leads to a change in the amplitude of contractions to a certain frequency stimulation. What we detected was an enhancement of the amplitude even after 0.1 or 1 μM capsaicin, which led us to consider the possibility of a differential depletion of neuropeptides by such doses of capsaicin resulting in a relatively lower concentration of relaxing than constricting neuropeptides in the local sensory nerve endings. The highest dose of capsaicin (10 μM) did not give rise to a significant change in the amplitude of the contractions; presumably it caused an irreversible damage in the sensory nerves [35].

The most abundant relaxing neuropeptide in the sensory nerves of the rat uterus is CGRP. A majority of the receptors for CGRP are located in the myometrium and the remainder in the endometrium. Although the receptor density rises as the gestation proceeds, the receptor numbers are not significantly different in nonpregnant and term pregnant rats [36]. In late but not term pregnancy (approximately days 18–20), however, the myometrial CGRP receptor number is doubled. It is presumed that CGRP is mainly responsible for the maintenance of pregnancy by ensuring a relaxant state of the myometrium. Here when the uteri of intact nonpregnant rats were stimulated at low frequency (1–30 Hz) in the presence of 10 μM CGRP receptor antagonist hCGRP(8-37), an enhancement of the amplitude of the contractions was detected, compared with the contractions evoked in the absence of hCGRP(8-37) at the same frequencies. These findings support the idea that CGRP-containing nerves participate in the uterine contractions induced by low-frequency (<30 Hz) electric field stimulation. In contrast, several previous reports have indicated that higher frequency stimulation is required for the activation of CGRP-containing nerves and the elicitation of relaxation in different smooth muscles, e.g., trachea [27, 28], ileum [29], or mesenteric arteries [30]. However, in our experiments the amplitudes of the contractions in the presence of 10 μM hCGRP(8-37) were highest when the uteri were stimulated at 1 Hz. Such low-frequency impulses to activate CGRP-containing nerves have been reported to elicit antidromic vasodilation [37], in which CGRP is the key modulator. The augmentation of the contraction response elicited by 1 Hz in the presence of 10 μM hCGRP(8-37) is interesting because the amplitude approaches that detected at the same frequency after the uteri were incubated with 1 μM capsaicin in vitro. This supports our suggestion that the increased amplitude of responses after in vitro capsaicin treatment is a consequence of, at least partially, the capsaicin-induced release and consequent depletion of CGRP in the sensory nerve endings, as was seen at 1 Hz.

Our immunohistochemical findings support a role of CGRP in the mechanism of this phenomenon. Indeed, CGRP-IR nerve fibers were sparse after a prior exposure to capsaicin at the lower concentrations of 0.1 and 1 μM. CGRP-IR nerves were practically absent after pretreatment with capsaicin at a concentration of 10 μM. These observations support our suggestion that changes in the tissue levels of CGRP play a significant role in the mechanism of the capsaicin-induced augmentation of uterine contractile responses.

The effect of capsaicin pretreatment on neurally evoked contractions was also investigated in the uteri of term pregnant rats. A remarkable difference from nonpregnant uteri was the lack of responses at 1- to 10-Hz stimulation. Our previous studies already disclosed the influence of pregnancy-induced denervation process on the elicitability of neurogenic contractions in the pregnant rat uterus [38]. In the late pregnant rat uterus, the lack of contraction responses to electric field stimulation at 1–10 Hz appeared in parallel with the progressive loss of uterine adrenergic fibers detected by histofluorescence [39]. Thus, it was not surprising that the uteri of term pregnant rats can not be stimulated at such low frequencies.

Our current findings suggest that CGRP-containing nerves in the nonpregnant uterus are possibly stimulated at 1–10 Hz. The lack of contractile responses to low frequency stimulation in term pregnant rats may be explained by a partial degeneration of CGRP-containing afferent nerves at that period of pregnancy. The contraction responses in term pregnant rat uteri to stimulation at 20–70 Hz also proved to be higher after incubation with 0.1 or 1 μM capsaicin, similarly as in the nonpregnant rat uteri, which supports the notion that certain populations of sensory nerves in the uterus [21] do not degenerate with pregnancy. Our findings pointed to the significance of capsaicin-sensitive afferent nerves in the uterine motor activity. The increased amplitude of uterine contractions after an in vitro incubation with capsaicin may be a result of an integrative mechanism that involves an altered neuropeptide content, a relative deficiency of CGRP, in the sensory nerve endings. Pregnancy-induced denervation might likewise affect afferent nerves [40, 41], possibly involving CGRP-containing nerves. The augmentation of the responses on high-frequency stimulation cannot be attributed to an involvement of CGRP. Our preliminary experimental data, showing a relative sparing of GAL-IR nerve fibers following an in vitro exposure to capsaicin suggest that increased contractile responses to high-frequency stimulation may be related to an effect of GAL. Indeed, GAL is abundant in reproductive tissues of the female rat, participates in the regulation of the reproductive process, and causes contractions of the myometrium [42]. However, only a detailed quantitative immunohistochemical analysis could reveal the exact nature of the population(s) of capsaicin-sensitive nerves involved in the mechanism of this response.

Capsaicin proved to be a suitable pharmacological tool

to investigate the contribution of sensory pathways to the uterine motor activity. This natural vanilloid substance has been used routinely to identify capsaicin-sensitive pathways and to explore their contribution to physiological and pathological regulatory processes [13]. Through the administration of capsaicin, the activation of sensory nerves and a consecutive neuropeptide release can be evoked. A similar sequel can also be triggered by local inflammatory processes: the activation of sensory nerves by low pH via VR1 and/or acid-sensing ion channels or by local proteases via protease-activated receptors [43] is involved in the mediation of nociception and inflammation. We assume that the change in the peptide transmitter content and release, and in particular the depletion of CGRP caused by sensory nerve activation, is a possible mechanism of increased uterine contractility, evoked by inflammation of the genital tract. We suggest that these mechanisms may contribute to the enhancement of the contractile activity of the uterus leading to premature birth.

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