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Characterization of capsaicin-induced, capsazepine-insensitive relaxation of ileal smooth muscle of rats

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Abstract

The mechanisms underlying the capsaicin-induced relaxation of the acetylcholine- as well as KCl-contraction were studied by measuring isometric force and phosphorylation of 20-kDa regulatory light chain subunit of myosin (MLC_{20}) in ileal longitudinal smooth muscles of rats. Capsaicin relaxed acetylcholine- and KCl-stimulated preparations in a concentration-dependent manner; the former was less sensitive to capsaicin than the latter and maximum responses to capsaicin (a percentage of papaverine-induced relaxation) were 70.6 ± 7.5%, $n = 10$ and 97.1 ± 0.9%, $n = 13$, $P < 0.05$, respectively. The response showed no desensitization. Like nifedipine, capsaicin relaxed the tissue precontracted with an agonist of L-type Ca^{2+} channels as well. The relaxant effect of capsaicin was not inhibited by capsazepine (a selective antagonist of vanilloid VR1 receptors), nitro-L-arginine, indomethacin, guanethidine, nor by inhibitors of soluble guanylate cyclase. Capsaicin inhibited acetylcholine-induced transient contraction in a Ca^{2+}-free, EGTA solution. Phosphorylation of MLC_{20} (a percentage of phosphorylated to total MLC_{20}) was increased 1 min after application of 10 μM acetylcholine (7.8 ± 2.0%, $n = 6$ vs. 22.6 ± 3.2%, $n = 6$) and of 65.9 mM KCl (2.2 ± 0.3%, $n = 8$ vs. 10.7 ± 1.7%, $n = 12$). Capsaicin reduced the KCl-induced increase more markedly than acetylcholine-induced increase in MLC_{20} phosphorylation. When the tissue was contracted for 20 min with acetylcholine, MLC_{20} phosphorylation was increased, and capsaicin reduced markedly the contraction and abolished MLC_{20} phosphorylation both elicited by acetylcholine. It is suggested that capsaicin relaxes the rat ileum via its direct action on smooth muscle, and that capsaicin inhibits contractile mechanisms involving extracellular Ca^{2+} influx via non-L-type Ca^{2+} channels, possibly via store-operated Ca^{2+} channels and Ca^{2+} release from intracellular storage sites. The effects of capsaicin on acetylcholine- and KCl-induced contraction could be explained by a decrease in MLC_{20} phosphorylation.

Keywords: Capsaicin; Capsazepine; Ca^{2+} influx; Myosin light chain phosphorylation

1. Introduction

Capsaicin, a pungent constituent of hot peppers, has become a widely accepted tool for studying sensory neuron function in the peripheral nervous system, since it specifically acts through capsaicin receptors (vanilloid VR1 receptors) on C and Aδ primary afferents to produce pain and warmth (Maggi and Meli, 1988). The receptors are a nonselective cation channel with relatively high permeability to Ca^{2+} in the cell membrane and the excitation by capsaicin of sensory neurons is due to influxes of mono- and divalent cations (Caterina et al., 1997). Capsaicin also activates Ca^{2+} release from intracellular Ca^{2+} stores via vanilloid VR1 receptors (Eun et al., 2001; Marshall et al., 2003). The capsaicin-sensitive primary afferents induce the release of neuropeptides from their peripheral endings, eliciting pronounced responses in gastrointestinal smooth muscles (Szallasi and Blumberg, 1999). A typical hallmark of specific pharmacological effects of capsaicin on the smooth muscles is their rapid desensitization, and capsazepine is a competitive antagonist for vanilloid VR1 receptors (Maggi et al., 1993; Liu et al., 1998). Well, capsaicin exerts contractile or relaxant effects on the smooth muscles, depending on the species, tissues and experimental protocols. For instance, under resting tension, capsaicin causes contraction in guinea pig ileum, which

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is mediated via sensory neurons (Barthó et al., 1982, 1999), while in agonist-stimulated human, rat and guinea pig intestine, it elicits vanilloid VR1 receptor-mediated relaxation (Maggi et al., 1986, 1990; Barthó et al., 1987, 1991, 2002; Giuliani et al., 1991).

Capsaicin also may have its action on neurons other than primary afferents and directly on smooth muscles, as to say, certain actions of capsaicin seem to be unrelated to its agonist action on vanilloid VR1 receptors in primary afferents (Szallasi and Blumberg, 1999). For instance, relaxant responses of guinea pig ileum, colon and stomach to capsaicin at certain concentrations are unaffected by previous exposure of the tissues to capsaicin, suggesting a nonspecific (or a vanilloid VR1 receptor-independent) smooth muscle depressant effect (Barthó et al., 1987; Maggi et al., 1987; Barthó and Holzer, 1995; Sim et al., 2001). The vanilloid VR1 receptor-independent relaxation has also been demonstrated in human and equine airways (Ellis et al., 1997; Zhu et al., 1997) and rat aorta (Lo et al., 1995; Yeon et al., 2001). These investigators have found that the relaxation is due to an activation of Ca^{2+}-activated K^+ channels or an inhibition of voltage-dependent L-type Ca^{2+} channels. Release of arachidonic acid from neuroendocrine cells produced by capsaicin is also capsazepine-insensitive (Someya et al., 2002). The biological relevance of these findings is unclear, and mechanisms of the capsaicin-induced, vanilloid VR1 receptor-independent relaxation in intestinal smooth muscle continue to be debated.

Smooth muscle contraction is regulated by intracellular Ca^{2+} and involves phosphorylation of the 20-kDa regulatory light chain subunit of myosin (MLC_{20}) by myosin light chain kinase, and MLC_{20} phosphorylation allows activation of myosin adenosine triphosphatase by actin, resulting in contraction (Somlyo and Somlyo, 1994). By contrast, agonist-induced contraction in vascular smooth muscle is maintained despite a fall in intracellular Ca^{2+} and MLC_{20} phosphorylation, suggesting an increase in Ca^{2+} sensitivity of contractile protein and MLC_{20} phosphorylation-independent contraction (Himpens et al., 1990). Further, myosin light chain kinase can stimulate the adenosine triphosphatase activity of smooth muscle myosin without phosphorylating MLC_{20}; MLC_{20} phosphorylation-independent contraction may involve phosphorylation of certain proteins (Walsh et al., 1994; Rembold et al., 2000).

The present study made use of rat longitudinal ileum, since the tissue responded to capsaicin by capsazepine-insensitive relaxation; this relaxation has received less attention and intestinal tracts are highly heterogeneous. Therefore, the present study was designed to characterize the relaxant action of capsaicin as follows; (1) the effects of capsaicin were examined on contraction produced by an agonist and high-KCl solution and on agonist-induced contraction in Ca^{2+}-free solution and (2) it was determined whether or not capsaicin altered the MLC_{20} phosphorylation produced by the agonist and KCl.

2. Materials and methods

2.1. Ileal preparations and tension measurement

The experimental protocols were in accordance with the guidelines of The Japanese Pharmacological Society. Male Wistar rats (230–330 kg) were anesthetized with pentobarbital (40 mg/kg, i.p.) and then exsanguinated. A 20–15-cm-long segment of the intestine proximal to the ileocecal valve was removed, the contents expelled by flushing the lumen with a Krebs–Henseleit bicarbonate (KHB) solution (composition in mM: NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and dextrose 10). A longitudinal incision was made along the mesenteric border in 5-cm-long segments, and the longitudinal muscle strip was prepared by peeling, with a cotton stick, the outer layer of the segment along lines of cleavage. The preparation (length: 1.5–2 cm, width: 2.5–3 mm) was suspended between two metal pins in 20 ml of warmed (37 °C) and oxygenated (95% O_2 and 5% CO_2) KHB solution for isometric recordings. The load on the preparations was 400 mg. After the equilibration period of 90 min, the preparation was contracted three to four times with 10 μM acetylcholine and 65.9 mM KCl for 10–20 min at 40-min intervals.

2.2. Effects of capsaicin on acetylcholine- and KCl-induced contraction

The preparation was contracted twice with 10 μM acetylcholine or 65.9 mM KCl for 20 min at 75-min intervals. In addition, two cumulative concentration–response curves for KCl were made with an interval of 90 min between each determination. The second contraction was made 15–20 min after and during treatment with capsaicin (0.3–30 μM).

2.3. Relaxant responses to capsaicin and nifedipine

The preparations were contracted with 10 μM acetylcholine and 65.9 mM KCl. After the contraction had reached steady state, capsaicin at concentrations of 0.1–100 μM was added to the KHB solution; only one concentration in each preparation, otherwise described. When relaxant response to capsaicin became maximum, papaverine (0.1 mM) was further added to obtain the maximum relaxation. One of paired preparations was treated with 5 μM capsazepine, 100 μM nitro-L-arginine, 3 μM guanethidine, 10 μM indomethacin, 10 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one) and 10 μM methylene blue for 30–60 min, and another untreated preparation was used as control. In some experiments, the tissue was incubated with capsaicin (30 and 100 μM) for 30–45 min and was then washed every 10 min for 80 min. After this period, the relaxant effect of capsaicin was evaluated in the tissue precontracted with acetylcho-

line. Concentration–response relationships for nifedipine and capsaicin were also determined in the tissues precontracted with a combination of 15 mM KCl and 0.3 μM Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester), which contracted the tissue to the similar extent as 65.9 mM KCl did.

2.4. Effect of nifedipine on capsaicin-induced relaxation in depolarized tissue

The preparation which had been contracted with 65.9 mM KCl was relaxed to baseline tensions by adding 100 nM nifedipine and again contracted with acetylcholine to obtain a contraction similar to that elicited with acetylcholine (10 μM) alone; concentrations of acetylcholine which was varied to obtain a comparable increase in tone in each preparation were 22.5 ± 1.8 μM, $n = 12$. After the contraction had reached steady state, capsaicin (3 and 30 μM) was added to the KHB solution. When the response to capsaicin became maximum, papaverine-induced relaxation was further obtained as described elsewhere.

2.5. Effect of capsaicin on acetylcholine-induced contraction in Ca^{2+}-free solution containing 0.6 mM EGTA

The preparation was contracted for 5 min with 10 μM acetylcholine, three times at 60-min intervals in the normal KHB solution. The tissue was treated for 15 min with 1, 3, 30 and 100 μM capsaicin or 100 nM nifedipine in the normal KHB buffer and then contracted with 10 μM acetylcholine before and 2 min after the preparation was exposed to a Ca^{2+}-free solution containing 0.6 mM EGTA with the same concentrations of capsaicin or nifedipine. The results are expressed as a percentage of the contractions elicited by acetylcholine in the normal KHB solution or in the KHB solution containing capsaicin or nifedipine.

2.6. Measurement of MLC_{20} phosphorylation

The ileal strips were contracted with 10 μM acetylcholine or 65.9 mM KCl. Within 15 s after the tissue had been relaxed for 15 min with 0.3, 3 and 30 μM capsaicin, the tissue was quick-frozen with 10% trichloroacetic acid in acetone-dry ice containing 10 mM dithiothreitol and allowed to reach room temperature. The tissue was then washed with acetone for 5 min to remove residual trichloroacetic acid. Proteins, including MLC_{20}, were extracted in urea-glycerol-polyacrylamide gel electrophoresis (PAGE) sample buffer (8 M urea, 20 mM Tris, 23 mM glycine, 10 mM dithiothreitol, 0.004% bromophenol blue and saturated sucrose, pH 8.6) for 60 min by sonication. Nonphosphorylated and phosphorylated forms of MLC_{20} were separated by urea-glycerol-PAGE followed by electrophoretic transfer of the proteins to nitrocellulose membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline overnight at 4 and incubated with anti MLC_{20} antibody for 3 h at room temperature. After incubation with the secondary antibody, blots of nonphosphorylated and phosphorylated MLC_{20} were detected with the enhanced chemiluminescence (Amersham Pharmacia Biotech, Tokyo). The blots of the enhanced chemiluminescence were quantitated by Lumi-Imager™ F1 (Roche Diagnostic, Germany). The extent of MLC_{20} phosphorylation is expressed as a percentage of phosphorylated forms to total MLC_{20}. Protein concentrations were determined using Bradford-dye-binding assay (Bio-Rad).

2.7. Drugs and solutions

The following drugs were dissolved in distilled water and diluted with the KHB solution; acetylcholine chloride (Sigma, St. Louis, MO, USA), DL-dithiothreitol (Sigma), guanethidine monosulfate (Sigma), methylene blue (Katayama Chem., Tokyo), N^G-nitro-L-arginine (Peptide Institute, Minoh, Japan), papaverine HCl (Wako), and trichloroacetic acid (Wako). Capsaicin (Sigma), capsazepine (Sigma) and 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Sigma) were dissolved in dimethyl sulfoxide (Sigma), and indomethacin (Sigma), nifedipine (Sigma) and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester ((±)-Bay K8644, Sigma), were dissolved in ethanol. The Ca^{2+}-free solution was prepared by removing $CaCl_2$ from the normal KHB solution and adding ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA, Sigma) at a final concentration of 0.6 mM. Anti-MLC_{20} antibody was purchased from Sigma, and anti-mouse immunoglobulin M peroxidase-labelled antibody (Biosource International, CA) was used as a secondary antibody.

2.8. Statistical analysis

Acetylcholine- or KCl-induced contraction in the presence of capsaicin is expressed as a percentage of that in the absence of capsaicin, and capsaicin-induced relaxation is expressed as a percentage of papaverine-induced relaxation. Potency of the relaxants is expressed as a negative log EC_{50} value, where the EC_{50} value is the molar concentration producing 50% of the maximum agonist response. Data are presented as mean values ± S.E. of the number (n) of observations. The one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference was used. Statistically significant differences were assumed when the P value was less than 0.05 with Student's t-test for two mean values and with the one-way analysis of variance followed by Fisher's protected least significant difference for multiple means.

3. Results

3.1. Effects of capsaicin on acetylcholine- and KCl-induced contraction

In basal conditions, rat ileal preparations were quiescent. Acetylcholine and KCl at 10 μM and 65.9 mM, respectively, produced a phasic, followed by a tonic (sustained) increase in tension in the preparation (Fig. 1A and D). The amplitude of contraction at 1 and 20 min after application of acetylcholine was 757 ± 68 and 560 ± 59 mg (n = 12), respectively, and that for KCl was 641 ± 58 and 552 ± 83 mg (n = 13), respectively. Capsaicin at 0.3 μM for 15 min did not inhibit the acetylcholine-induced contraction, but the drug at 1–30 μM reduced the amplitude of contraction at 1 and 20 min after application of acetylcholine in a concentration-dependent fashion (Fig. 1B and C). The amplitude of contraction at 1 and 20 min after 65.9 mM KCl was reduced by capsaicin at 0.3 to 30 μM (Fig. 1E and F). Capsaicin at 3 μM nearly abolished the KCl-induced contraction but just partly inhibited the acetylcholine-induced contraction. Thus, the KCl-induced contraction was more susceptible to capsaicin than that elicited by acetylcholine.

Cumulative concentration–contractile response curves for KCl were shifted to the right and downward by capsaicin (0.3, 1 and 3 M) (Fig. 2A).

Capsaicin did not contract the preparations under present conditions.

3.2. Relaxant effects of capsaicin and nifedipine

To observe relaxant response, the preparation was contracted with a submaximal concentration of acetylcholine (10 μM). Capsaicin did not relax the precontracted tissue at 0.1 μM, but relaxed it at higher concentrations (1–100 M) in a concentration-related manner (Fig. 2B). The relaxant response did not show tachyphylaxis; the tissue was relaxed by capsaicin (30 and 100 μM), and the drug was washed out by replacing repeatedly the fresh KHB solution for 80 min. Capsaicin at the same concentrations caused relaxation to the similar extent as

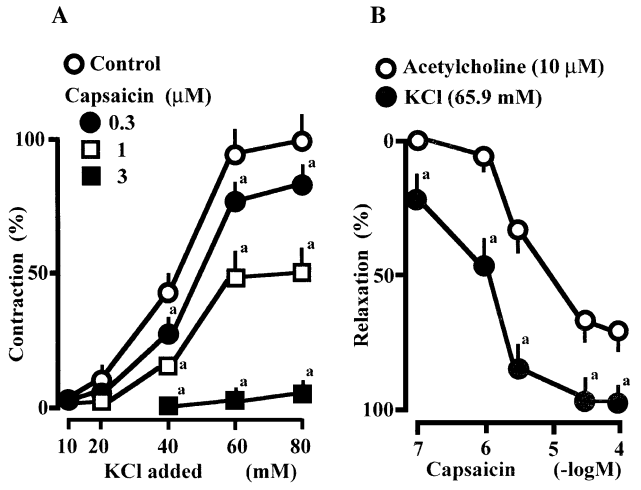

Fig. 2. [A] Cumulative concentration–response curves for KCl-induced contraction. The curves were determined 15–20 min after and during treatment with capsaicin at 0.3 (●), 1 (□) and 3 (■) μM. Control (○). Ordinate; the maximum KCl response in the first concentration–response curve is expressed as 100% (626 ± 94 mg, n = 18). [B] Single concentration–response curves for capsaicin-induced relaxation in ileal smooth muscles precontracted with 10 μM acetylcholine (○) or with 65.9 mM KCl (●). Ordinate, the relaxation due to papaverine is expressed as 100%. [a]Significantly different from control [A] or acetylcholine-treated tissues [B] (P < 0.05).

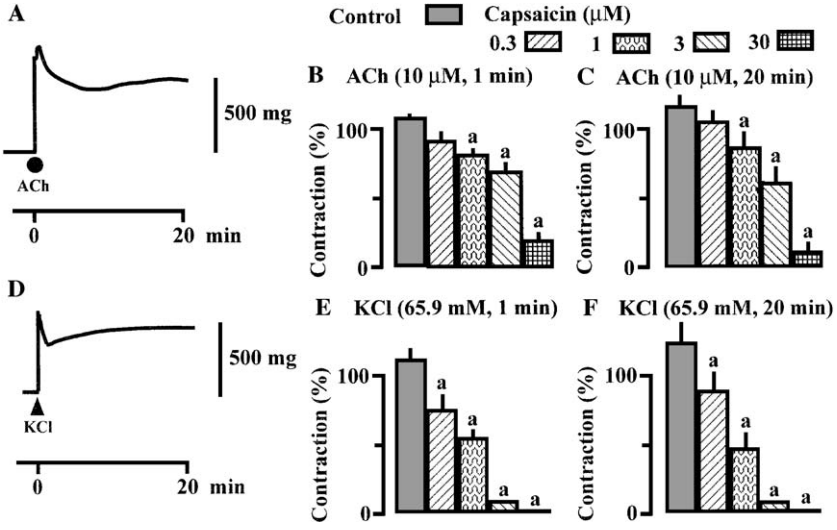

Fig. 1. Effects of capsaicin (0.3–30 μM) on acetylcholine—[A, B, C] and KCl—[D, E, F] induced contraction of ileal smooth muscle. [A, D] Acetylcholine (ACh, 10 μM) and KCl (65.9 mM) elicited a phasic, followed by a tonic contraction. Horizontal and vertical lines represent time (min) after application of the contractile drugs to the muscle and tension calibration (500 mg), respectively. The amplitude of the contraction elicited at 1 and 20 min after application of acetylcholine [B, C] or KCl [E, F] was determined. Ordinate, a percentage of the amplitude of the (first) contraction in the absence of capsaicin. Results are expressed as means ± S.E. (n = 6–8). [a]P < 0.05 vs. control.

did the first capsaicin (data not shown). In addition, prior treatment of the tissue with 30 and 100 μM capsaicin for 30–45 min, followed by washing capsaicin out from the organ bath solution, did not impair the ability of the tissue to relax in response to 30 μM capsaicin.

Tissues, which had been contracted with a submaximal concentration of KCl (65.9 mM), were more sensitive to 0.1 μM capsaicin than the acetylcholine-stimulated tissue (Fig. 2B). Capsaicin at 100 μM could relax the acetylcholine- and KCl-stimulated preparations to 70.6 ± 7.5%, $n = 10$ and 97.0 ± 7.5%, $n = 13$, $P < 0.05$, respectively, of the papaverine-induced relaxation. The absolute values of papaverine-induced relaxation in the acetylcholine- and KCl-stimulated preparations were not significantly different; 863 ± 144 mg, $n = 10$ and 955 ± 121 mg, $n = 13$, respectively. Capsazepine (5 μM), nitro-L-arginine (100 μM), guanethidine (3 μM), indomethacin (10 μM), ODQ (10 μM) and methylene blue (10 μM) did not change the capsaicin (3–10 μM)-induced relaxation in the tissues precontracted with either acetylcholine or KCl (data not shown).

Next, the preparation was contracted with a combination of 15 mM KCl and 0.3 μM Bay K8644 (an L-type Ca^{2+} channel agonist) to similar extent as with 65.9 mM KCl alone. Like nifedipine, capsaicin (0.3–30 μM) relaxed the precontracted tissue in a concentration-dependent fashion (Fig. 3A). Capsaicin was less potent as a relaxant than nifedipine;-log EC_{50} values, 5.85 ± 0.21, $n = 4$ vs. 7.15 ± 0.10, $n = 4$, $P < 0.05$. Like capsaicin, nifedipine was a more potent relaxant in KCl- than acetylcholine-contracted preparations; $-\log EC_{50}$ values, 8.55 ± 0.28, $n = 5$ vs. 7.70 ± 0.18, $n = 4$, $P < 0.05$ (Fig. 3B). In some experiments, the ileum was contracted with 65.9 mM KCl and the contraction was reversed by 100 nM nifedipine. The tissue was again contracted with acetylcholine (22.5 ± 1.8 μM, $n = 12$) after the tension reached the basal level in the presence of nifedipine. Under these conditions, capsaicin (3 and 30 μM) elicited a relaxation to lesser extent than in the tissue precontracted with KCl alone, and the relaxant response to

Table 1
Effects of capsaicin on acetylcholine-induced contraction of rat ileum in Ca^{2+}-free solution containing 0.6 mM EGTA

Drug	Concentration (μM)	n	Acetylcholine (%)
Control		25	23.1 ± 4.1
Capsaicin	1	5	11.2 ± 2.4[a]
	3	5	6.9 ± 2.2[a]
	30	5	2.6 ± 2.2[a]
	100	5	1.9 ± 0.3[a]
Nifedipiine	0.1	5	20.1 ± 4.7

The tissue was contracted with 10 μM acetylcholine, 2 min after the tissue was exposed to a Ca^{2+}-free, EGTA solution. Results are percentages of acetylcholine-induced contraction in the Ca^{2+}-free solution relative to their corresponding contractions obtained in the normal KHB solution.

[a] $P < 0.05$ compared to control.

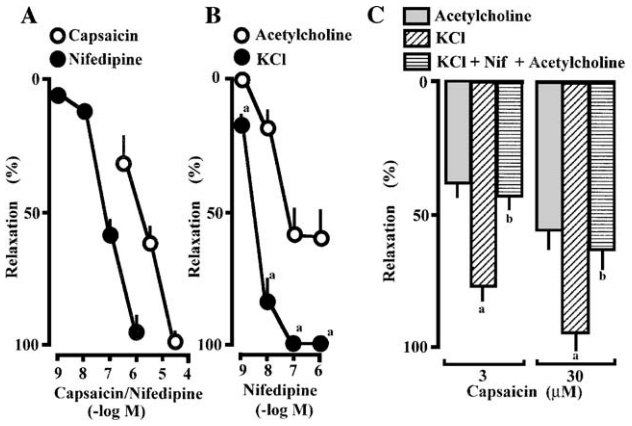

Fig. 3. [A] Single concentration–response curve for capsaicin (○)- and cumulative concentration–response curve for nifedipine (●)-induced relaxation in ileal tissues precontracted with a combination of 15 mM KCl and 0.3 μM Bay K8644. Ordinate, papaverine-induced relaxation is expressed as 100% (529 ± 128 mg, $n = 16$). [B] Cumulative concentration–response curves for nifedipine-induced relaxation in acetylcholine (10 μM, ○)- and KCl (65.9 mM, ●)-stimulated tissues. Ordinate; absolute values of the amplitude of relaxation in response to papaverine (100%) were 565 ± 169 mg ($n = 4$) and 508 ± 102 mg ($n = 5$) in the acetylcholine- and KCl-stimulated preparations, respectively. [C] Effect of nifedipine on capsaicin (3 and 30 μM)-induced relaxation in the ileal preparation contracted with acetylcholine in the presence of 65.9 mM KCl. The preparation was contracted with 65.9 mM KCl and the contraction was then reversed by nifedipine (Nif, 100 nM) to baseline tensions and thereafter again contracted with acetylcholine at concentrations of 22.5 ± 1.8 μM ($n = 12$). Absolute values of the amplitude of the papaverine-induced relaxation (100%, ordinate), were 819 ± 119 mg ($n = 6$) in acetylcholine-treated group, 769 ± 71 mg ($n = 5$) in KCl-treated group and 796 ± 94 mg ($n = 6$) in nifedipine-treated group. Significantly different from acetylcholine-induced contraction[a] and KCl-treated group[b] ($P < 0.05$).

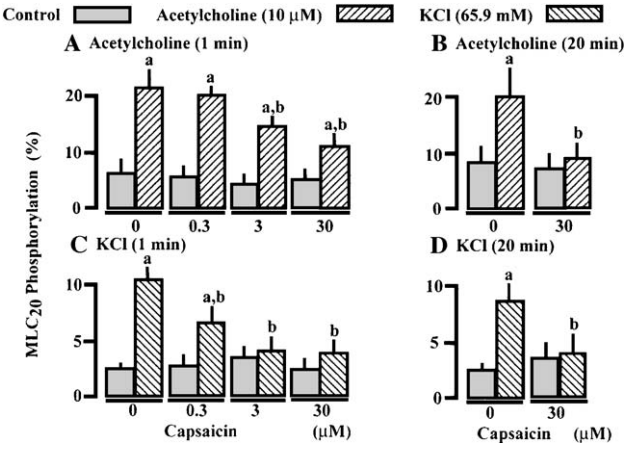

Fig. 4. Inhibitory effect of capsaicin on increase in MLC_{20} phosphorylation produced with acetylcholine or KCl. The preparation was incubated for 15 min with 0.3, 3 or 30 μM capsaicin, and thereafter the preparation was incubated for another 1 and 20 min with 10 μM acetylcholine [A, B] or 65.9 mM KCl [C, D] in the continuous presence of the same concentrations of capsaicin. Ordinate, a percentage of phosphorylated forms to total MLC_{20}. Vertical bars represent S.E. of means ($n = 4–7$). Significantly different from controls[a] and from acetylcholine (or KCl) in the absence of capsaicin[b] ($P < 0.05$).

capsaicin was actually the same whether KCl plus nifedipine were present or not (Fig. 3C).

3.3. Effects of capsaicin and nifedipine on acetylcholine-induced contraction in a Ca^{2+}-free solution containing 0.6 mM EGTA

The preparation was first contracted with 10 μM acetylcholine in the normal KHB solution (100%, 933 ± 102 mg, $n = 25$). Then, when the preparation was incubated for 2 min in a Ca^{2+}-free solution containing 0.6 mM EGTA, acetylcholine (10 μM) elicited a short-lived, phasic contraction. When the preparation was exposed for 15 min to 1, 3, 30 and 100 μM capsaicin in the KHB solution and then contracted with 10 μM acetylcholine, 2 min after the tissue was exposed to the Ca^{2+}-free solution with capsaicin at the same concentrations, capsaicin reduced the contraction induced by acetylcholine in a concentration-related manner (Table 1). When the tissue was treated with nifedipine (100 nM) instead of capsaicin, the drug did not significantly alter the acetylcholine-induced contraction in the Ca^{2+}-free solution. KCl (65.9 mM) did not evoke an apparent contraction in the Ca^{2+}-free solution (data not shown).

3.4. Effects of capsaicin on acetylcholine- and KCl-induced phosphorylation of MLC_{20}

Acetylcholine (10 μM) increased phosphorylation of MLC_{20} (6.3 ± 2.5%, $n = 6$ vs. 21.5 ± 3.3%, $n = 6$, $P < 0.05$) at 1 min (Figs. 4A and 5A). Prior treatment with capsaicin at 0.3 μM, a concentration which did not significantly alter the acetylcholine-induced contraction, did not change the increase in MLC_{20} phosphorylation produced by acetylcholine. Capsaicin (30 μM) seemed to reduce the basal levels of MLC_{20} phosphorylation (Fig. 5A), but it was not true statistically (Fig. 4A). On the other hand, capsaicin (3 and 30 μM) for 15 min significantly reduced the acetylcholine-induced MLC_{20} phosphorylation (Figs. 4A and 5A). When the tissue was treated for 20 min with 10 μM acetylcholine, acetylcholine caused the increase in MLC_{20} phosphorylation (Figs. 4B and 5B). The acetylcholine-induced increase in MLC_{20} phosphorylation was abolished by 30 μM capsaicin.

KCl (65.9 mM) increased MLC_{20} phosphorylation (2.5 ± 0.3%, $n = 4$, vs.10.5 ± 1.1%, $n = 4$, $P < 0.05$) at 1 min (Fig. 4C). The KCl-induced increases in MLC_{20} phosphorylation was inhibited by 0.3 μM and nearly abolished by 3 μM capsaicin. Thus, capsaicin altered the KCl-induced increase in phosphorylation of MLC_{20} more markedly than that produced by acetylcholine. KCl for 20 min increased phosphorylation of MLC_{20} (Fig. 4D). Capsaicin at 30 μM abolished KCl-induced phosphorylation of MLC_{20}.

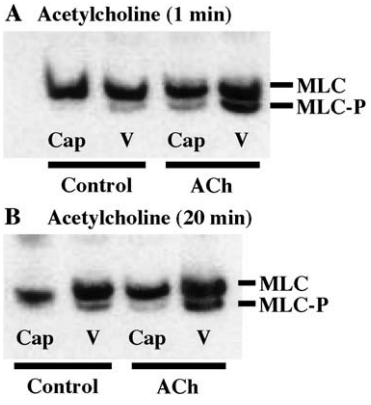

Fig. 5. Representative immunoblotting of MLC (nonphosphorylated MLC_{20}) and MLC-P (phosphorylated MLC_{20}) using anti-MLC_{20} antibody. The tissues were treated with vehicle (V) and 30 μM capsaicin (Cap) for 15 min and then contracted for 1 min [A] or 20 min [B] with 10 μM acetylcholine (ACh).

4. Discussion

Capsaicin has been known to contract and/or dilate (relax) gastrointestinal preparations, and some of these actions of capsaicin and its related compounds (vanilloids) have been explained by the liberation of sensory neuropeptides from the peripheral endings of (vanilloid-sensitive) primary sensory neurons (Szallasi and Blumberg, 1999). The present and previous studies showed that capsaicin did not modify resting tone of longitudinal muscle from rat and human intestines (Maggi et al., 1990; Nocerino et al., 2002). Namely, relaxation was the only apparent effect of capsaicin in the rat ileum (similar preparations from guinea pigs responded to capsaicin by contraction and relaxation, which were and were not mediated by vanilloid VR1 receptors, respectively, unpublished data). The relaxation did not show tachyphylaxis and was not modified by capsazepine, capsaicin pretreatment nor by guanethidine, suggesting that the relaxation was not accounted for by an activation of vanilloid VR1 receptors on sensory neurons nor an activation of adrenergic neurons, but may reflect its direct or nonspecific action on smooth muscle cells. Supporting this, the relaxant effect of capsaicin was obtained at concentrations higher than a few μM, concentrations at which capsaicin could activate vanilloid VR1 receptors (Maggi et al., 1990; Barthó and Holzer, 1995). The nonspecific depressant effect of capsaicin (1–100 μM) on smooth muscle from various species was also reported (Maggi et al., 1987; Barthó et al., 1987; Barthó and Holzer, 1995; Lo et al., 1995; Ellis et al., 1997; Zhu et al., 1997; Yeon et al., 2001). However, the possibility should not be excluded that capsazepine-insensitive isoform of vanilloid VR1 receptors on sensory neurons participated in the relaxant response (Liu et al., 1998). Although there was evidence suggesting an involvement of nitric oxide, cyclic GMP and prostanoids in capsaicin-induced relaxation of smooth muscle (Wood et al., 1989; Manzini, 1992; Barthó and Holzer, 1995; Szarek et al., 1998; Barthó et al., 2002), the present study with

inhibitors of nitric oxide synthase, soluble guanylate cyclase and cyclooxygenase (nitro-L arginine, ODQ, methylene blue and indomethacin) showed that these factors were not responsible for the capsaicin-induced relaxation.

The relaxant response of the KCl (65.9 mM)-stimulated ileum to capsaicin was more marked than that of the acetylcholine-stimulated tissue. Again, the response of the KCl-stimulated ileum to capsaicin was not mediated by capsazepine-sensitive vanilloid receptors, nitric oxide, cyclic GMP, prostanoids nor by adrenergic nerves. The relaxant response to capsaicin on acetylcholine-induced contraction was actually the same, whether both nifedipine (100 nM) and KCl (65.9 mM) were present or not. We also found that capsaicin (30 μM) as well as nifedipine (1 μM) could abolish the contractile response to a combination of 15 mM KCl and 0.3 μM Bay K8644, an L-type Ca^{2+} channel agonist which promotes Ca^{2+} influx without depolarization of the membrane. These findings suggest that changes in membrane potential itself do not affect the capsaicin-induced, vanilloid VR1 receptor-independent relaxation, capsaicin may rather inhibit extracellular Ca^{2+} influx, possibly through store-operated Ca^{2+} channels. Capsaicin was also suggested to inhibit Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Maggi et al., 1987; D'Alonzo et al., 1995; Lo et al., 1995; Sim et al., 2001; Nocerino et al., 2002), and the relaxation was due to decrease intracellular Ca^{2+} (Yeon et al., 2001). Since capsaicin is lipid-soluble, it is possible that the drug penetrates the cell membrane to act directly on lipid bilayer of the Ca^{2+} stores, and there is evidence that capsaicin regulates Ca^{2+} release from intracellular Ca^{2+} stores in the primary sensory neurons (Eun et al., 2001; Marshall et al., 2003). Therefore, we investigated effects of capsaicin on acetylcholine-induced contraction in the absence of extracellular Ca^{2+}. Acetylcholine-induced phasic contraction in the Ca^{2+}-free solution was inhibited by capsaicin but not by nifedipine, and high-KCl solution did not contract the preparation in the Ca^{2+}-free solution. These results suggest that the contraction does not depend upon Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels and capsaicin reduces the contraction involving Ca^{2+} release from intracellular storage sites. Alternatively, capsaicin could change the sensitivity of contractile proteins to Ca^{2+}.

Phosphorylation of MLC_{20} regulates smooth muscle contraction, and an activation of myosin light chain kinase is largely dependent upon intracellular concentrations of free Ca^{2+} (Somlyo and Somlyo, 1994). Therefore, we investigated whether or not capsaicin reduced MLC_{20} phosphorylation. Capsaicin might reduce basal levels of MLC_{20} phosphorylation (Fig. 5A), but it was not the case statistically. KCl increased MLC_{20} phosphorylation, and capsaicin reduced the KCl-induced force development and MLC_{20} phosphorylation. Capsaicin at 30 μM completely abolished the responses to KCl. It is suggested that the inhibition by capsaicin of the KCl-induced contraction can be explained by the decrease in MLC_{20} phosphorylation, although we could not exclude the possibility that capsaicin caused a nonspecific inhibition of MLC_{20} phosphorylation produced by KCl. On the other hand, capsaicin reduced the acetylcholine-induced contraction and phosphorylation of MLC_{20} less potently than the responses to KCl. MLC_{20} phosphorylation increased by 20-min incubation with acetylcholine was completely abolished by capsaicin, but the contraction due to acetylcholine was not. It seems likely that the acetylcholine-induced contraction in the presence of capsaicin is independent of MLC_{20} phosphorylation. Thus, the existence of MLC_{20} phosphorylation-independent component is suggested to account for the less marked effects of capsaicin in the acetylcholine- than KCl-stimulated tissues. Certain enzymes including protein kinase C participate in the MLC_{20} phosphorylation-independent contraction, which may involve phosphorylation (or dephosphorylation) of certain proteins including caldesmon, calponin and heat shock protein (Walsh et al., 1994; Throckmorton et al., 1998; Rembold et al., 2000). Ratz et al. (2002) found that capsaicin did not affect an agonist-induced, protein kinase C-mediated contraction in rabbit stomach. However, characterization of the contractile response of rat ileum to acetylcholine in the presence of capsaicin remains to be determined.

Since the concentrations of capsaicin used in the current experiments were obviously higher than those used for studying vanilloid VR1 receptor-mediated responses, it seems unlikely that the effects of capsaicin observed here reflect some physiological event. However, we expect that capsaicin and its analog becomes a good tool for studying mechanisms underlying contraction and relaxation of smooth muscle.

In conclusion, capsaicin reduced the contractile response to KCl more markedly than those to acetylcholine. Capsaicin-induced relaxation did not undergo desensitization and was not mediated through capsazepine-sensitive vanilloid VR1 receptors. Capsaicin inhibited contractile mechanisms involving extracellular Ca^{2+} influx, possibly via store-operated Ca^{2+} channels and Ca^{2+} release from acetylcholine-sensitive storage sites. Capsaicin decreased acetylcholine- and KCl-induced increases in MLC_{20} phosphorylation. It is suggested that the relaxant effect of capsaicin is due to a decreased phosphorylation of MLC_{20}.

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