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BRADYKININ-INDUCED CONTRACTIONS OF BOVINE MESENTERIC LYMPHATICS

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SUMMARY

1. The mode of action of bradykinin (BK) on bovine mesenteric lymphatics was investigated by recording isometric tensions and action potentials in the isolated longitudinal segments.

2. Addition of BK in concentrations from 10^{-10} to 4×10^{-6} M caused dose-related tonic contractions.

3. BK in a low concentration accelerated the rhythm of action potential discharges in the spontaneously beating preparations and elicited frequent discharges of action potentials and a rapid rise in smooth muscle tone associated with phasic contractions.

4. BK in high concentrations (more than 10^{-7} M) caused a further rise of tension in the preparations which had already been depolarized in a high-K solution.

5. The contraction induced by 4×10^{-9} M-BK in the standard solution was abolished in a Ca-free environment or in the presence of a Ca-antagonist, 10^{-4} M-D-600, though more than 50 % of the contraction caused by 10^{-6} M-BK still remained in both circumstances.

6. In a Ca-free solution containing 1 mM-EGTA (Ca-free standard solution), 10^{-6} M-BK caused a slight contraction even after high-K-induced contractions were completely blocked.

7. The contractile response to 10^{-6} M-BK in the Ca-free standard solution was augmented after activation of β-receptors.

8. It is concluded that the BK-induced contractions may be closely related to an increased Ca influx through the membrane and release of membrane-bound and intracellular Ca. The increased uptake of Ca into the BK-sensitive intracellular store may contribute to the relaxing effect of β-agonist.

INTRODUCTION

Changes in activity of lymphatic smooth muscle affect significantly the rate of lymph transport (Ohhashi, Azuma & Sakaguchi, 1980). Recently we have successfully recorded membrane action potentials of bovine mesenteric lymphatics simultaneously with phasic contractions which had one-to-one correspondence to the action potentials

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(Azuma, Ohhashi & Sakaguchi, 1977). Ca current was thought to play a determining role in producing spike discharges in the lymphatic smooth muscle. Activation of intramural sympathetic nerves by a short train of electrical pulses elicited a rise in tone of the lymphatic smooth muscle (Ohhashi & Roddie, 1981). Contractile responses of the lymphatics were also induced by vasoactive agents, such as 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$, noradrenaline, histamine, dopamine and acetylcholine (Ohhashi, Kawai & Azuma, 1978). The lymphatics were particularly sensitive to 5-hydroxytryptamine. Several investigators have demonstrated constrictive or dilatatory effects of bradykinin (BK) on various arteries and veins (Tsuru, Ishikawa & Shigei 1974; Toda, 1977). No report, however, has been made of the effect of BK on lymphatic smooth muscle.

We investigated the mode of action of BK on bovine mesenteric lymphatics by recording isometric tensions and action potentials in the isolated segments. The Ca dependence of the BK-induced response in the lymphatics was also studied to analyse the characteristics of excitation–contraction coupling in the smooth muscle.

A preliminary account of this work has been communicated to the Physiological Society (Ohhashi & Roddie, 1980).

METHODS

Lymphatics, about 2 mm in outer diameter, were dissected from fresh mesentery obtained as soon as possible (usually 20–30 min) after the cattle had been slaughtered. Segments, 20 mm long, were prepared from the isolated lymphatics after removal of surrounding adipose tissue in Krebs solution at 37 °C to prevent the fat from solidifying and making gentle dissection impossible. Each of these segments was vertically fixed between hooks in a 10 ml organ bath filled with Krebs solution circulating through a heat exchanger kept at 37 °C. The composition of Krebs solution (standard solution) (in mM) was as follows: NaCl, 120·0; $NaHCO_3$, 25·0; KCl, 5·9; NaH_2PO_4, 1·2; $CaCl_2$, 2·5; $MgCl_2$, 1·2 and glucose 5·5. The solution was equilibrated before and during the experiment with a gas mixture of 95% O_2 + 5% CO_2 in the organ bath to give a pH of 7·4. Hooks anchoring the upper and lower ends of the segment were connected to the lever of a force-displacement transducer (Statham model UC3) and the bottom of the bath, respectively. The longitudinal tension detected by the transducer was amplified and recorded on Device M2 pen recorder. The resting tension of each segment was set at 200 mg, which had been found to be optimum for obtaining a maximum contractile response in a lymphatic vessel having an outer diameter of 2 mm (Ohhashi & Roddie, 1981). All preparations were allowed to equilibrate for 60–90 min in the oxygenated bathing medium before the start of experiments.

Cumulative dose–response relationships of BK triacetate (Sigma) were obtained in the presence or absence of various drugs: i.e. phentolamine mesylate (Ciba-Geigy), isoproterenol hydrochloride (Nikken Kagaku), propranolol hydrochloride (Sumitomo Kagaku), atropine sulphate (Tanabe Seiyaku), tetrodotoxin (Sigma), methysergide hydrogen maleate (Sandoz), diphenhydramine hydrochloride (Sigma), indomethacin (Sigma) and a Ca antagonist, D-600 hydrochloride (Knoll AG). All drugs, except for BK, were freshly diluted in the standard Krebs solution. The doses are expressed in terms of the base and at the final organ bath concentration.

Contractile responses of the lymphatic segments to BK were examined not only in the standard bathing medium but also in a high-K solution, which had the composition (mM): KCl, 125·9; $KHCO_3$, 25·0; KH_2PO_4, 1·2; $CaCl_2$, 2·5; $MgCl_2$, 1·2 and glucose 5·5.

In experiments in which the role of Ca in the contractile response of lymphatic smooth muscle to BK was explored, the standard bathing solution was modified so as to have the following composition (mM): NaCl, 120·0; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; HEPES (Sigma), 11·5 and glucose 5·5. The solution was adjusted to pH 7·4 with HCl. Ca excess solutions were made merely by adding appropriate amounts of $CaCl_2$ to this modified standard solution. To examine the possible effect of hypertonicity produced by an increase in $CaCl_2$, a hypertonic solution was prepared which

contained 10 % sucrose, with the other ions in the standard solution being unchanged. No apparent effect of hypertonicity was observed on the basal tone of the lymphatic segments. Ca-deficient solutions were obtained by substituting equi-osmolar amounts of NaCl for $CaCl_2$ in the modified standard solution. To the Ca-free solution thus prepared, was added 1 mM of EGTA, i.e. ethyleneglycol-bis (*β*-aminoethylether)-*N*, *N'*-tetraacetic acid (Ca-free standard solution). A high-K solution with no Ca^{2+} (Ca-free high-K solution) was obtained by substituting equimolar amounts of KCl for NaCl in this Ca-free standard solution.

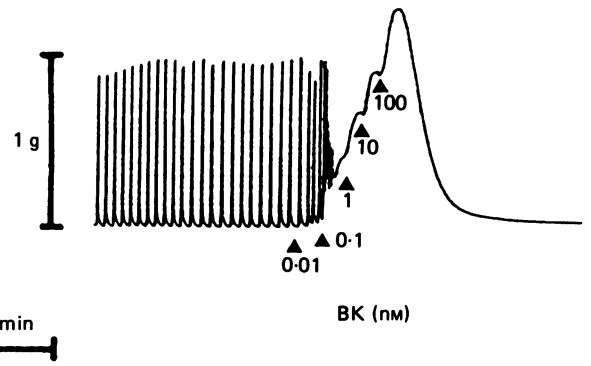

Fig. 1. Typical responses of a lymphatic preparation with spontaneous contractions to bradykinin (BK) ranging from 10^{-11} to 10^{-7} M in concentration.

Longitudinal strips, 40 mm long and 2 mm wide, were also prepared from the isolated lymphatics and mounted in a single sucrose-gap apparatus for simultaneous recording of electrical and mechanical activities. One end of each mounted strip was connected via a thin thread to a force-displacement transducer (Shinko Tsushin UL-10-120). This part of the strip was continuously superfused with the standard bathing solution or with one of the test solutions containing BK at various concentrations. These solutions were kept at 37 °C and continuously bubbled with a gas mixture of 95 % O_2 + 5 % CO_2 to give a pH of 7·4. Another end was anchored at a fixed point and depolarized by the high-K solution described above. The middle section of the strip was superfused with an isotonic sucrose solution. Non-polarizing Ag–AgCl wire electrodes, which were connected to a high input resistance pre-amplifier (Nihon Koden MEZ-8101), were placed in the conducting solutions bathing each end of the strip. The outputs from the electrodes and the mechanoelectric transducer were displayed on a dual-beam synchroscope (Iwatsu DS-5015) and recorded by a direct-writing oscillograph (Sanei Sokki 8S).

Results shown in the text and Figures are expressed as mean value ± S.E. of the mean. Statistical analyses were made using the paired or unpaired Student's *t* test and differences in means considered significant when $P < 0.05$.

RESULTS

Contractile responses to BK

Fig. 1 demonstrates typical responses of a lymphatic preparation with spontaneous contractions to BK, the concentration of which was elevated from 10^{-11} to 10^{-7} M. At the lowermost concentration, the spontaneous contraction rhythm of the preparation increased in association with a slight reduction in contraction amplitude. An elevation of BK concentration from 10^{-11} to 10^{-10} M induced a remarkable acceleration of the rhythm accompanied by a steep decrease in the amplitude. Then the preparation shifted to a state of sustained tonic contraction. Dose-dependent rises in tone of the lymphatic smooth muscle were induced by BK in concentrations more

than 10^{-9} M. These contractile responses were observed regularly in twenty-seven of thirty lymphatic vessels.

In preparations without spontaneous activity the administration of BK (10^{-10}–10^{-7} M) caused dose-related tonic contractions (Fig. 2*A*). Spontaneous contractions of small amplitude were occasionally elicited at a low concentration (10^{-11}–10^{-10} M). As shown in Fig. 2*A*, the contractile responses to BK were slightly less in the second trial than in the first. No appreciable change in the magnitude of the responses, however, was observed after the second trial. Hence, the cumulative dose–response relationship obtained in the second or subsequent trial was used as a control in experiments with a blocking agent or Ca antagonist.

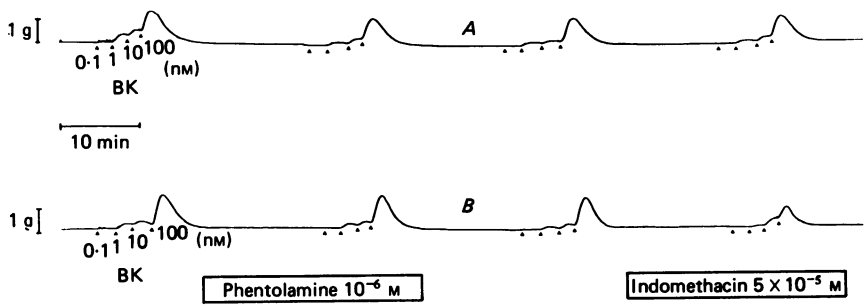

Fig. 2. *A*, typical cumulative dose–response relationships of BK (10^{-10}–10^{-7} M) in a lymphatic preparation without spontaneous activity. *B*, effects of phentolamine (10^{-6} M) and indomethacin (5×10^{-5} M) on the cumulative dose–response curve of BK in a quiescent preparation.

TABLE 1. Effects of pre-treatment with drugs on BK-induced contractions of lymphatic smooth muscle ($n = 5$)

	Developed tension (mg) (mean ± S.E. of mean)	
Concentration of BK	10^{-9} M	10^{-7} M
Control	142 ± 41	1192 ± 104
Pre-treatment		
Phentolamine (10^{-6} M)	128 ± 62	1142 ± 121
Propranolol (10^{-6} M)	101 ± 83	1098 ± 211
Atropine (10^{-6} M)	114 ± 71	1124 ± 186
Tetrodotoxin (10^{-7} M)	140 ± 74	1164 ± 124
Methysergide (10^{-6} M)	128 ± 55	1148 ± 162
Diphenhydramine (10^{-6} M)	134 ± 72	1107 ± 158

Fig. 2*B* shows a typical recording of the effects of phentolamine and indomethacin on the cumulative dose–response curve of BK in a quiescent preparation. Phentolamine (10^{-6} M) had no effect on the response. Indomethacin in a dose of 5×10^{-5} M, on the other hand, suppressed the response in a non-competitive manner. This drug decreased the maximum response to 10^{-7} M-BK by 32·8 ± 6·4 % (from 1298 ± 164 to 872 ± 123 mg, $n = 5$). The tension of lymphatic smooth muscle developed in the high-K solution was also reduced by 5×10^{-5} M-indomethacin by 29·6 ± 4·8 % (from 1154 ± 192 to 812 ± 186 mg, $n = 5$). Table 1 summarizes the effects of pre-treatment with other

blocking drugs on the contractile responses of the lymphatic segments to BK. Each drug was administered 15 min before the application of BK. None of these drugs affected the contractions induced by BK in low (10^{-9} M) and high (10^{-7} M) concentrations.

Effects of BK on electrical and mechanical activities

Simultaneous recordings of electrical and mechanical activities in lymphatic smooth muscle were made by use of the single sucrose-gap technique (Fig. 3). In the lymphatic preparations without spontaneous activity, BK in a low concentration (4×10^{-9} M) elicited a slight depolarization superimposed by frequent discharges of action potentials. These electrical changes were accompanied by a rapid rise in smooth muscle tone. The declining phase of the contraction curve was superimposed by phasic

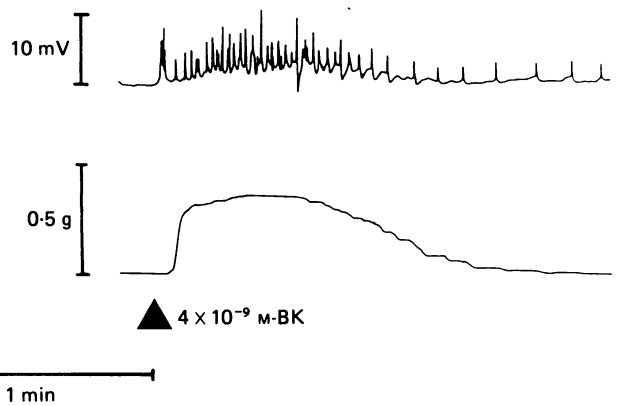

Fig. 3. Effects of a low concentration of BK (4×10^{-9} M) on electrical (upper tracing) and mechanical (lower tracing) activities in lymphatic smooth muscle recorded by use of the single sucrose-gap technique.

undulations which had one-to-one correspondence to the action potentials. In the lymphatic preparations with spontaneous activity, the administration of BK in a low concentration (10^{-11} M) accelerated the rhythm of action potential discharges. The level of resting potential was seldom affected by the administration of BK, with the exception of a few cases in which a transient slight depolarization took place at an initial stage.

Effects of a Ca antagonist and environmental Ca^{2+} concentration

Fig. 4 shows cumulative dose–response relationships of BK in the quiescent lymphatic preparations before (open circles) and after (filled circles) pre-treatment with a Ca antagonist, D-600 (10^{-4} M). Each curve was depicted by using the mean values of five experiments. The abscissa is the concentration of BK in logarithmic scale. The ordinate is the isometric tension expressed in a value relative to that developed by 4×10^{-6} M-BK in the standard bathing solution. The control relationship may be divided into three phases, i.e. the initial slow ascent in a concentration range from 10^{-10} to 4×10^{-8} M, the intermediate steep ascent in a concentration range from 4×10^{-8} to 10^{-6} M, and the final saturation phase in concentrations above 10^{-6} M.

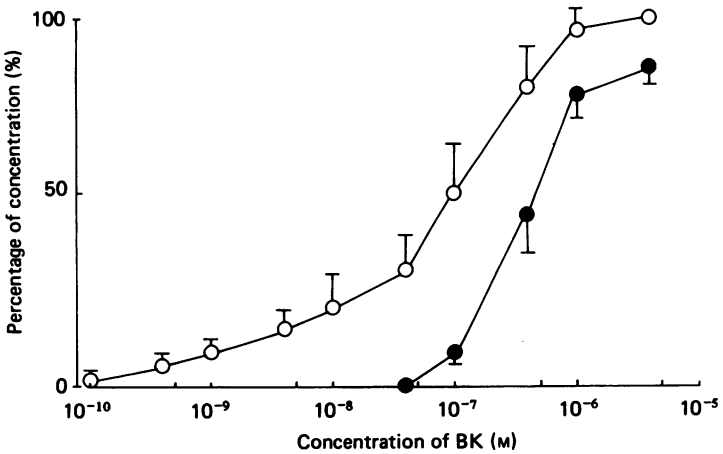

Fig. 4. Cumulative dose–response relationships of BK in quiescent lymphatic preparations before (○—○) and after (●—●) pre-treatment with a Ca antagonist, D-600 (10^{-4} M). Data are presented as mean ±S.E. of the mean ($n = 5$).

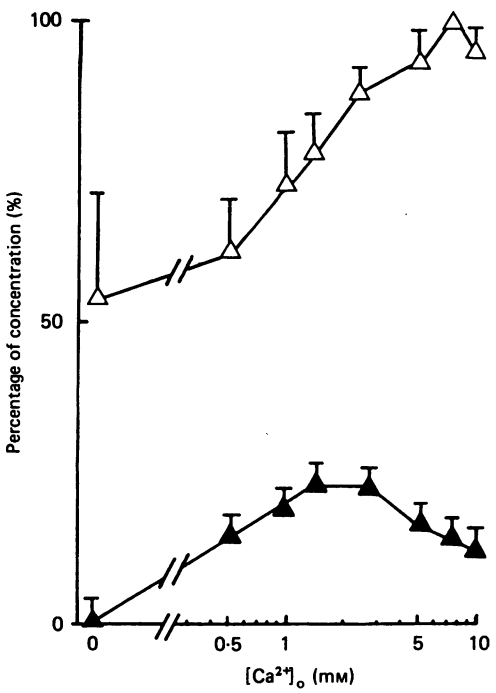

Fig. 5. Relationships between extracellular concentration of Ca^{2+} and the magnitude of contractions induced by a high (10^{-6} M; △—△) and low (4×10^{-9} M; ▲—▲) concentrations of BK in quiescent lymphatic preparations. The magnitude is expressed in a value relative to the maximum tension developed by 10^{-6} M-BK in the presence of external Ca concentration, $[Ca^{2+}]_o$, of 8 mM. Data are presented as mean ±S.E. of the mean ($n = 5$).

Pre-treatment with 10^{-4} M-D-600 shifted the curve to the lower right. Most of the initial phase disappeared, since the threshold concentration rose from 10^{-10} to 4×10^{-8} M. The maximum tension developed by 4×10^{-6} M-BK was about 85 % of that in the control.

The extracellular concentration of Ca^{2+} was plotted against the magnitude of contraction induced by BK (Fig. 5). The magnitude was expressed in a value relative to the maximum tension developed by 10^{-6} M-BK in the presence of 8 mM-Ca^{2+}. Two curves were depicted by using the average values of five experiments each. Filled triangles demonstrate the relationship when the lymphatic smooth muscle was activated with BK in a low concentration (4×10^{-9} M). The contraction did not occur in the Ca-free solution. The magnitude of contraction increased with increasing extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$) and attained a maximum value at 1·5 mM.

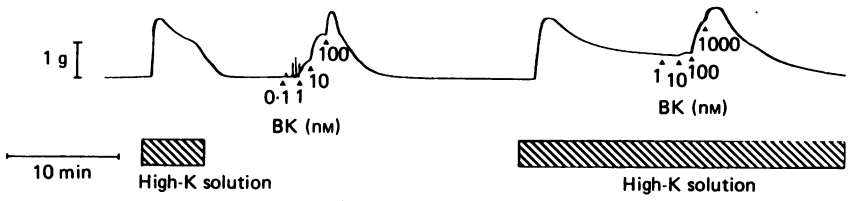

Fig. 6. K contracture and BK-induced contraction. Left half: typical responses of a lymphatic preparation to the high-K solution and to the administration of BK ($10^{-10}-10^{-7}$ M). Right half: a typical cumulative dose–response relationship of BK (10^{-9}–10^{-6} M) obtained with the lymphatic preparation in a state of K contracture.

A dose-dependent inhibition of the BK-induced contraction took place at $[Ca^{2+}]_o$ of more than 3 mM. Open triangles show the results when a high concentration (10^{-6} M) of BK was used. In contrast to the above-mentioned relationship, more than half of the maximum tension still remained in the Ca-free solution. The magnitude of contraction increased with increasing $[Ca^{2+}]_o$ up to 8 mM.

Effects of membrane depolarization

The effect of BK was investigated on the lymphatic preparations which were completely depolarized by raising the external concentration of K (Fig. 6). Replacement of the standard bathing solution with the high-K solution produced a sudden rise in tension, which declined gradually to a certain fixed level (K contracture). It has already been demonstrated that the membrane of lymphatic smooth muscle is depolarized completely in the isotonic high-K solution (Ohhashi & Azuma, 1982). Typical responses of lymphatic smooth muscle to the K depolarization and to administration of BK are shown in the left half of Fig. 6. The maximum tension developed by 10^{-7} M-BK was greater than that by K depolarization.

As shown in the right half of Fig. 6, the cumulative dose–response relationship of BK was obtained with the lymphatic preparations that had been in a state of K contracture. Dose-dependent contractions were still evoked by BK in concentrations higher than 10^{-8} M. The maximum tension achieved with 10^{-6} M-BK was greater than that achieved at an initial stage of the high-K administration.

Effect of removal of extracellular Ca^{2+}

The role of the intracellular Ca^{2+} store in the BK-induced contraction was studied with the lymphatic preparations incubated in the Ca-free standard solution containing 1 mM-EGTA (Fig. 7). Two deflexions shown in the left half of the Figure are the responses of a preparation to the high-K solution and BK, respectively. When the preparation was placed in a Ca-free environment, the BK-induced contractions were

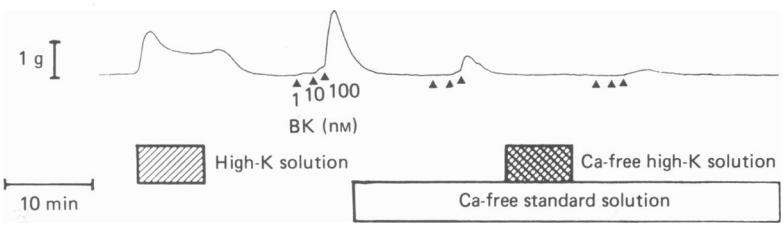

Fig. 7. BK-induced contractions in the Ca-free standard solution. Left half: typical responses of a lymphatic preparation to high-K solution and BK (10^{-9}–10^{-7} M). Right half: typical responses of the preparation to the Ca-free high-K solution and BK (10^{-9}–10^{-7} M). Right half: typical responses of the preparation to the Ca-free high-K solutions and BK (10^{-9}–10^{-7} M) in the Ca-free standard solution.

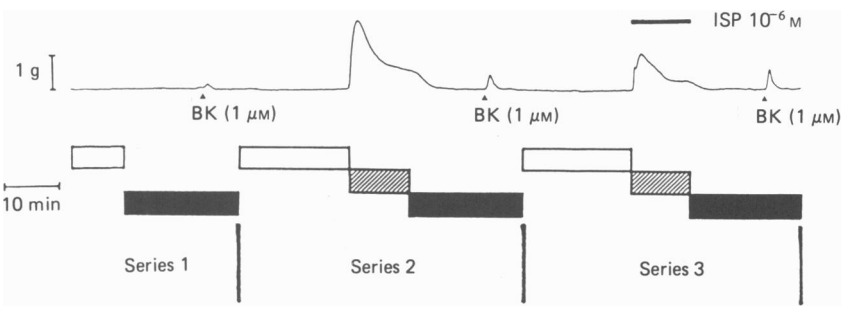

Fig. 8. Effects of pre-treatment with the high-K solution and isoproterenol (ISP) on BK-induced contraction in the Ca-free standard solution. □, the standard solution; ■, the high-K solution; ■, the Ca-free standard solution.

reduced in magnitude with time. After the incubation in the Ca-free standard solution for 18 min, no contraction was elicited by the replacement of the bathing medium with the Ca-free high-K solution. A slight contraction, however, could still be induced by BK in a high concentration after the Ca-free incubation for 30 min. These findings indicate that the contractions induced by BK in high concentrations (above 10^{-7} M) are more resistant to Ca^{2+} depletion than the contracture caused by the high-K environment.

The height of the contraction induced by 10^{-6} M-BK in the Ca-free standard solution was influenced by pre-treatment with the high-K solution with or without a β-agonist, 10^{-6} M-isoprenaline (Fig. 8). Each lymphatic preparation was incubated in the standard solution for 20 min at the start of each series of experiments ($n = 5$). A small contraction was elicited by 10^{-6} M-BK administered 15 min after the

replacement of the standard solution with the Ca-free standard solution (series 1). K contracture was induced by the application of the high-K solution for 10 min after the preceding incubation in the standard solution. The same amount of BK was given 15 min after changing the bathing medium from the high-K to the Ca-free standard solution (series 2). The BK-induced contraction in series 2 was greater than that in series 1. The intensity of K contracture was reduced by the combined use of the high-K solution and 10^{-6} M-isoprenaline. The BK-induced contraction after the combined use, however, was greater than that in series 2 (series 3). Changes in duration of the combined use from 3 to 10 min did not affect the magnitude of the BK-induced contraction in series 3.

DISCUSSION

BK-induced contraction of lymphatic smooth muscle

BK has been known to be a potent vasoactive substance which produces different effects on different parts of the vascular system. The nonapeptide is also related closely with the initiation and development of inflammation. It is a substance which raises the permeability of capillaries and possibly also of venules in the irritated area, thereby increasing the rate of production of interstitial fluid and lymph. Stürmer & Cerletti (1967) showed that infusion of BK (5–80 ng/kg . min) into the femoral artery of anaesthetized dogs produced a dose-dependent rise in the flow of lymph in the hind limb. The increase of lymph flow has been considered to be due to an elevation of lymph production resulting from increased capillary surface area and permeability (Svensjo, Persson & Rutili, 1977; Granger, Richardson & Taylor 1979). It is worth noting that the rate of lymph flow may be augmented not only by an elevation of lymph production but also by a rise in lymphatic smooth muscle activity, since contractions of lymphatic smooth muscle may drive lymph centripetally in the presence of directional valves. The present study revealed that, in the longitudinal preparations of bovine mesenteric lymphatics, BK caused an acceleration of spontaneous rhythmicity at low concentrations (10^{-11}–10^{-10} M) and elicited dose-related contractions at high concentrations ($> 10^{-8}$ M). Recently we have demonstrated that lymphatic smooth muscle was particularly sensitive to 5-hydroxytryptamine (Ohhashi, Kawai & Azuma, 1978). The threshold concentration for BK was about 1/500th of that for 5-hydroxytryptamine. This high sensitivity to BK may be characteristic of lymphatic smooth muscle. The increase in frequency of spontaneous contraction induced by BK in extremely low concentrations (10^{-11}–10^{-10} M) may facilitate lymph flow in the living body.

BK-induced rises of lymphatic smooth muscle tone were not inhibited by pretreatment with α-adrenergic blocking agents, anti-histamics, and 5-hydroxytryptamine antagonists (Fig. 2 and Table 1). Thence, α-adrenergic, histaminergic, or serotonergic mechanisms seemed to have nothing to do with the contractile response of lymphatic smooth muscle to BK.

Indomethacin, a prostaglandin cyclo-oxygenase inhibitor, suppressed the contractile responses of lymphatic smooth muscle to BK and K depolarization. It has been postulated that the influx of Ca^{2+} into smooth muscle cells is inhibited by indomethacin in concentrations from 6 to 60×10^{-5} M (Northover, 1971). Starr & West (1966)

demonstrated a non-specific antagonism of anti-inflammatory agents against BK actions in the isolated mesenteric artery of guinea-pigs. We could not rule out the possibility that the observed suppression of the responses to BK was non-specific in nature. Another possibility is that the suppression may be related to a reduction in synthesis or liberation of prostaglandins which cause lymphatic smooth muscle to contract. According to our recent study, however, the possibility seems unlikely, since contractile responses of lymphatic smooth muscle to noradrenaline and 5-hydroxytryptamine were also inhibited by 20–40% after pre-treatment with indomethacin at the same concentration as shown in Fig. 2.

Ca dependence of BK-induced contraction

The finding that removal of extracellular Ca, or addition of a Ca antagonist, abolished responses to low concentrations but not to high concentrations of BK resembles similar observations made on other smooth muscles with other agonists (see Bolton, 1979 for review). It suggests that low concentrations of the agonist act by facilitating entry of Ca through the cell membrane but that high concentration can act by releasing Ca stores in the cell.

Another important aspect of this study is the action of a β-agonist on the BK-induced contraction in the Ca-free standard solution. Isoprenaline decreased the tension elicited by K depolarization (Fig. 8). This may indicate that the β-agonist enhances the uptake of intracellular Ca into the store when an increase in Ca influx due to K depolarization takes place. The enhanced storage of intracellular Ca may play a role in the potentiation of the BK-induced contraction after the combined application of high-K stimulation and isoprenaline. The observed inhibition of K contracture under the influence of isoprenaline may be due, at least in part, to an enhanced uptake of intracellular Ca into the store (Casteels & Raeymaekers, 1979).

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