Vol. 156, No. 1, 1988
October 14, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 186-192

ENDOTHELIN AND Ca^{++} AGONIST BAY K 8644: DIFFERENT VASOCONSTRICTIVE PROPERTIES

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Received August 25, 1988

The mechanism of vasoconstriction induced by endothelin was investigated in rat isolated aorta in comparison with the Ca^{++} agonist, Bay K 8644. Endothelin (EC_{50}=4 nM) induced a slow and sustained contraction in control medium whereas the one elicited by Bay K 8644 (EC_{50}=14 nM) necessitating a partly K^{+} depolarized medium was fast with superimposed rhythmic contraction. By opposition with Bay K 8644, endothelin contraction was not inhibited by the calcium antagonists (1 μM) nifedipine, diltiazem and D 600, and substantially persisted in Ca^{++} free medium or after depletion of intracellular Ca^{++} by phenylephrine (1 μM). These data show that endothelin does not act as an activator of potential dependent Ca^{++} channels but probably through specific receptor(s) as suggested by its mode of vasoconstriction.

Furchgott and Zawadzki (1) first reported that the relaxing effect of acetylcholine on rabbit aorta was mediated by substance(s) released from the endothelium : the endothelium derived relaxing factor(s) (EDRF). Since this original observation, extensive studies have focused on the role of endothelium in the regulation of the vascular smooth muscle. Recently, Yanagisawa et al. (2) have isolated and identified from porcine aortic endothelial cells a highly potent vasoconstrictor peptide termed endothelin. Considering the properties and the structure of this compound that presents some regional homologies with the amino acid sequence of neurotoxins active on ion channels, it was proposed that endothelin acts as an endogenous agonist of the dihydropyridine sensitive Ca^{++} channels (2). The dihydropyridine derivative Bay K 8644 diametrically opposes the action of calcium antagonists typified by nifedipine and is currently used as slow channel calcium activator (3).

In an attempt to define more precisely the vasoconstriction mechanism of endothelin, we investigated the contractile properties of endothelin in comparison with Bay K 8644 in rat isolated aorta.

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0006-291X/88 $1.50

MATERIALS AND METHODS

Male Sprague Dawley rats (Charles River, Paris, 270 g-360 g) were sacrificed by cervical dislocation and the thoracic aorta removed and cleaned of the surrounding tissue. Rings 2 mm wide were suspended in organ baths containing 10 ml of physiological solution (for composition see below) under a tension of 2 g at 37°C and gassed with 95% O_2 / 5% CO_2. Contractile responses were measured using force displacement transducers (Statham UC2) coupled to a Gould 8000 S polygraph. In some experiments the endothelium was mechanically disrupted by gently rolling a small forceps on the luminal surface of the rings. A 1 h equilibration period was allowed before experimentation.

Normal physiological solution was composed of (mM) : NaCl, 118 ; KCl, 4.7, $CaCl_2$, 2.5 ; KH_2PO_4, 1.2 ; $MgSO_4$, 1.2 ; $NaHCO_3$, 25 ; glucose, 11. The Ca^{++} free-medium, was prepared by substituting $CaCl_2$ with 1 mM EGTA and by reducing Mg_4SO_4 to 0.6 mM. After equilibration in normal medium the preparation was subjected to a near maximal dose (about 95%) of phenylephrine (PE, 1 µM). When the contraction was stable, carbachol (10 µM) was tested in order to test the integrity of endothelium (1).

After this sensitization different procedures were used :

- to study the contraction elicited by endothelin and Bay K 8644 a cumulative dose-response curve was constructed after a rest period of 45 min. In some case KCl (7.3 mM) was added five minutes before the beginning of this cumulative dose-response curve.
- to determine the effects of Ca^{++} channel antagonists four preparations from a given animal were used in parallel. One served as control whereas the three other received calcium antagonists introduced into the bath 30 min before agonist. The maximal contractile effect of each ring that had been subjected to antagonist was determined by comparing the response evoked by the application of sensitizing agent (PE) with that evoked in the control preparation. For experiments using Bay K 8644 as agonist KCl was increased to 12 mM five minutes before the beginning of the cumulative dose-response curve.
- to study the extracellular Ca^{++} dependence of endothelin response, the preparations were washed for 50 min in normal solution followed by 20 min in Ca^{++} free-medium. After agonists induced response $CaCl_2$ (2.5 mM) was added or tissues were washed in Ca^{++} free medium for an additional period of 20 min followed by a second addition of agonist.

Chemicals

Endothelin (human, porcine) corresponding to the 21 amino acid peptidic sequence, was purchased from Peninsula Lab. Inc. (Belmont CA, USA). EGTA, phenylephrine, Angiotensin II, carbachol, diltiazem and nifedipine were from Sigma Chemical Co. (St Louis MO, USA). Bay K 8644 was from Bayer A-G (Leverkusen, FRG) and D600 (gallopamil) was from Knoll A-G (Ludwigshafen, FRG).

Statistics

The data are given as means ± S.E.M. and one way analysis of variance (ANOVA) was used for statistical comparison of the results. CE_{50} were determined graphically.

RESULTS

Contractile responses of endothelin and Bay K 8644

In endothelium denuded rat aorta, the tone induced by endothelin was slow and sustained (Fig. 1). The one elicited by Bay K 8644 was fast with superimposed rhythmic contractions ; moreover high concentrations of Bay K

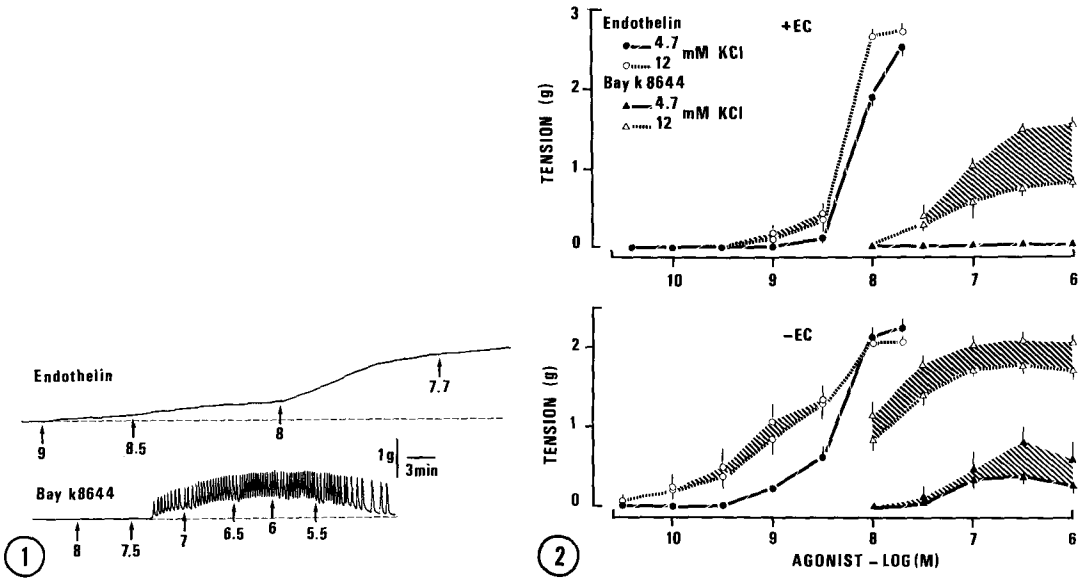

Figure 1 - Representative recordings of contraction produced by endothelin and Bay K 8644 in endothelium free rat aorta incubated in normal medium. Note rhythmic contractions elicited by Bay K 8644.

Figure 2 - Comparison of contraction elicited by endothelin and Bay K 8644 in rat aorta with (+EC, upper panel) and without (-EC, lower panel) endothelial cells. Preparations were incubated in normal (4.7 mM KCl) or partially depolarizing (12 mM KCl) solution. Hatched part indicates rhythmic contractions. Means ± SEM (n = 7).

8644 produced tissue relaxation (Fig. 1). The maximal tension observed with endothelin (2.2 ± 0.11 g, n = 7) was significantly higher than with Bay K 8644 (0.8 ± 0.22 g, n = 7, $p < 0.001$) (Fig. 2). Since full expression of Bay K 8644 required partial membrane depolarization (3,4,5) experiments were also carried out in partially depolarized arteries with K^+ (12 mM). In endothelium free preparations (Fig. 2, -EC), the sensitivity of both agonists was increased. The maximal tension elicited by Bay K 8644 was also greatly enhanced (2.1 ± 0.11 g, n = 7) reaching the level obtained with endothelin. In endothelium intact preparations (Fig. 2, +EC) Bay K 8644 failed to induce any contractile response in normal medium whereas in partly depolarizing medium, it produced a moderate tone superimposed with potent rhythmic contractions. The effect of endothelin was little affected by the presence of endothelium in normal medium or in depolarizing medium.

Effects of Ca^{++} channel antagonists

Fig. 3 shows that on endothelium free aorta Ca^{++} antagonists were very weak inhibitors of the contractile response induced by endothelin (EC_{50} = 4nM). Diltiazem (1 µM), D600 (1 µM) and nifedipine (1 µM) decreased the maximal tension induced by endothelin only by 8 ± 2.7%, 14 ± 1.3% and 16 ±

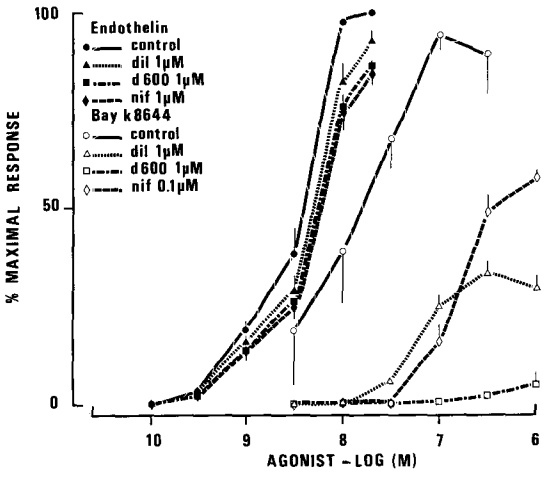

Figure 3 - Effects of diltiazem (dil), D 600 and nifedipine (nif) on the contraction induced by endothelin and Bay K 8644. Experiments using Bay K 8644 as agonist were conducted in partially depolarizing solution. Means ± SEM (n = 5).

2.5%, respectively (n = 5). In contrast, in partly depolarized endothelium free aorta, diltiazem (1 µM) reduced (66 ± 3.0% inhibition, n = 5) and D600 (1 µM) nearly abolished (95 ± 3.5% inhibition, n = 5), the contraction evoked by Bay K 8644 (EC_{50}=14 nM). As expected, nifedipine (0.1 µM) the dihydropyridine derivative shifted to the right the Bay K 8644 dose-response curve. These results are consistent with the mechanism of action of Bay K 8644 that depends upon influx of extracellular Ca^{++} through potential dependent channel (3,4,5,6).

Contractile response of endothelin in Ca^{++} free medium

Since the effect of endothelin appeared different from that of Bay K 8644, we studied the action of endothelin in Ca^{++} free medium. Phenylephrine (PE, 1 µM) induced a phasic contraction which was 27 ± 1.5% (n = 6) of the maximum response obtained in calcium containing solution. Endothelin also developed a slow and sustained contraction (29 ± 1.2%, n = 6 of the maximum PE response in control medium) (Fig. 4). After addition of Ca^{++} to the medium a tonic contraction was obtained (PE : 102 ± 4.4% and endothelin : 107 ± 3.8% of the maximal PE response in control medium).

Effects of repeated challenges with different agonists in Ca^{++} free-medium

Two consecutive exposures to PE (1 µM) in Ca^{++} free medium induced a decline in the contraction. This inhibition was unlikely due to a change in PE sensitivity since it was also observed if PE was replaced by angiotensin II (0.1 µM). Thus, a reduction of intracellular Ca^{++} available for release by PE or angiotensin II could be involved. After first exposure to PE, a second

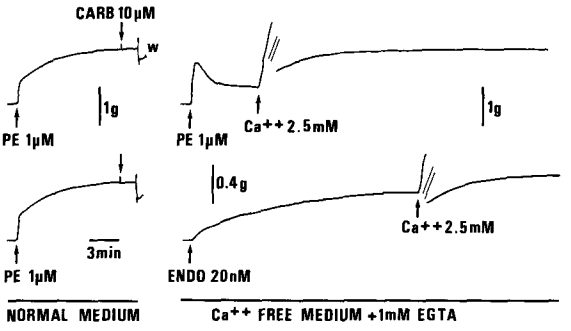

Figure 4 - Contractile response produced by phenylephrine (PE) in normal medium and by PE and endothelin (ENDO) in calcium free EGTA medium. Effect of Ca^{++} introduced to the bath after agonist response in Ca^{++} free medium. The break in the tension recordings denotes a change in scale. Note that, in normal medium, carbachol (CARB) did not induce relaxation of the PE precontracted ring, as there is no endothelium present.

challenge of PE reduced the contraction to 9.5 ± 1.29% (n = 6) of the maximal response of PE obtained in control medium whereas the contraction elicited by endothelin remained to the same 24 ± 1.4% (n = 6)(Fig. 5).

DISCUSSION

The present study reveals a clear dissociation between the contractile properties of endothelin and Bay K 8644 in isolated rat aorta. As previous described (3,4,5) the contraction elicited by Bay K 8644 is dependent on extracellular Ca^{++} in flux through potential dependent channels and is blocked by Ca^{++} antagonists. But as unexpected, Ca^{++} antagonists do not effectively affect the contraction induced by endothelin. Moreover, Bay K

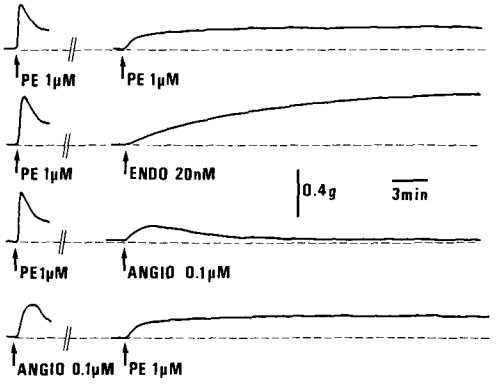

Figure 5 - Representative recordings of effect of repeated challenge with agonists : phenylephrine (PE), Angiotensin II (ANGIO) and endothelin (ENDO) in rat aorta incubated in a Ca^{++} free EGTA medium. Preparations were washed for 20 min in Ca^{++} free medium between the two agonist applications.

8644 requires a partial membrane depolarization which is not necessary to express full effect of endothelin. However, the potentiating effect of partial K^+ depolarization is attenuated for both agonists in presence of endothelium indicating a possible membrane hyperpolarization by EDRF (7,8).

Thus, it is unlikely that endothelin produces rat aorta contraction by a mechanism similar to Bay K 8644, promoting Ca^{++} influx through potential dependent channels as initially postulated (2). In vascular smooth muscle the contractile response induced by agonist in a Ca^{++} free medium can be separated into two components : a fast phase due to a mobilization of intracellular Ca^{++} stores and a tonic phase obtained after readmission of Ca^{++} to the medium which is dependent on extracellular Ca^{++} (9,10,11). Endothelin elicites a slow tonic contraction in rat aorta incubated in a Ca^{++} free medium and after calcium readmission a sustained contraction is obtained. This suggests that the endothelin contractile response consits of two components, one is independent of Ca^{++} influx, the other is dependent on Ca^{++} influx insensitive to potential dependent channel blockers. Regarding the extracellular Ca^{++} independent component the present study also shows that the endothelin contraction is much more resistant to Ca^{++} depletion than PE or angiotensin II. Since this component is probably due to intracellular Ca^{++} release, our results may indicate that endothelin releases Ca^{++} in higher concentration or from different intracellular stores than PE or angiotensin II. Alternatively, it may be postulated that endothelin like the phorbol esters increases the affinity of protein kinase C for Ca^{++} since slowly developing sustained contraction resistant to Ca^{++} depletion was also observed with these compounds (12,13,14). In addition the C-kinase inhibitor H-7 reversed the contraction induced by endothelin on porcine coronary artery (15).

Finally, these results suggest that endothelin acts through specific receptors different from the dihydropyridine recognition sites. Further studies are required to characterize these receptors.

Added Notes

The effects of endothelin presented here were also confirmed in our laboratory by studying $^{45}Ca^{++}$ influx in cultured smooth muscle cells and by C. Chander and D.A. Willoughby (personal communication) who found that isolated superfused rat myofibroblast preparation still contracts to endothelin in presence of Ca^{++} channel blockers and in a Ca^{++} free superfusion system.

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