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BLOCKADE OF ENDOTHELIN-INDUCED CONTRACTIONS BY DICHLOROBENZAMIL: MECHANISM OF ACTION

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Contractions of intact rat aortic rings induced by endothelin were totally inhibited by the amiloride analogue dichlorobenzamil (DCB) at concentrations known to block Na-Ca exchange (IC50=30 μM). Amiloride (100 μM) was ineffective. Ca-channel blockers and a K-channel opener elicited only partial inhibition. These results could indicate that the Na-Ca exchanger plays an important role in endothelin-induced contractions. Endothelin, however, had no effect on the kinetics of the exchanger, and, in addition, contractions also occurred in Na-depleted vessels. The endothelin-induced contractions produced by Ca release from intracellular pools were also completely inhibited by DCB. In fact, the latter compound was found to block contractions induced by Ca itself in the presence of Ca ionophore or detergent. We conclude that DCB acts directly on Ca-induced activation of myofilaments in smooth muscle.

Endothelin, a 21-amino-acid peptide recently isolated from the supernatant of cultured porcine endothelial cells, has been characterized as a very potent vasoconstrictor (1). The exact mechanism of its action is not yet well understood. In their original report, Yanagisawa et al. (1) suggested that endothelin acts like an endogenous agonist of the dihydropyridine-sensitive calcium channels. More recently, however, specific endothelin-binding sites have been found in vascular smooth-muscle cells (2). Calcium-entry blockers did not displace endothelin from its binding site and, conversely, endothelin did not interfere with the binding of calcium-entry blockers to their specific sites (2). These results indicate that the mechanism of action involved is different from that originally proposed (1). It was recently shown that endothelin activates the opening of non-specific cation channels, which induces sustained depolarization of the membrane and thus triggers the activity of the L-type Ca channels indirectly (3). In this study, we show that calcium-entry blockers only

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partially inhibit endothelin-induced contractions in rat aortic rings, whereas the Na-Ca-exchanger inhibitor dichlorobenzamil (DCB) blocked them totally. Experiments were carried out to investigate the mechanism of action of DCB. We found that the possible interference of DCB with Na-dependent Ca fluxes plays no part in endothelin-induced contractions. In fact, DCB even appeared to act at a level beyond the hormone-induced increase of intracellular Ca (either mobilized from intracellular pools or entering the cell via non-specific cation channels or L-type Ca channels). DCB prevented activation of the contractile apparatus in vascular smooth muscle by Ca ions.

MATERIALS AND METHODS

Rat thoracic aortic rings. The thoracic aorta was removed and cleaned of all loosely adherent connective tissue. From each aorta, four rings (about 2.5 mm wide) were cut close to the aortic arch. Intact rings were suspended under a tension of 1.5 g between two parallel hooks in a 20-ml organ-bath containing bicarbonate-buffered physiological salt solution (PSS) gassed with $95\%O_2/5\%CO_2$. After an equilibration period of 60 min, the artery preparations were contracted with noradrenaline (50 nM). At peak contraction, acetylcholine (0.5 µM) was added to check endothelial integrity (relaxation) (4). Contractile responses induced by endothelin were then studied in the absence or presence of the chosen concentration of different drugs, given 30 min, or in the case of dichlorobenzamil 60 min, before endothelin. Only one concentration per ring was used. Controls were exposed to an appropriate dilution of the solvent.

When the effect of low-sodium solution was studied, the experiments were performed in a modified PSS containing HEPES buffer (pH 7.4) gassed with 100% O_2, and low-sodium solutions (sodium 1.2 mM) were prepared by substituting choline chloride for NaCl. To study the effects of DCB on contractions induced by endothelin in the absence of extracellular calcium, rings were incubated for 15 min in a calcium-free PSS containing 2 mM EGTA.

In some experiments, the cell membrane was chemically removed by soaking the rings for 30 min in a skinning solution of the following composition: 136.9 mM KCl, 5.0 mM $MgCl_2$, 2.0 mM EGTA, 20 mM Tris-maleate, 5 mM ATP-Na_2 and 0.1mg/ml saponin at pH 6.8 and 22 °C. Calcium concentrations were changed by adding an appropriate amount of $CaCl_2$ to the bath (5).

Endothelin effect on the Na-Ca exchanger. Calf sarcolemmal membranes were prepared by the method of Jones et al. (6), as modified by Caroni et al. (7). At the end of the procedure the vesicles were equilibrated at a concentration of about 10 mg/ml in 160 mM NaCl, 20 mM HEPES, pH 7.4 (Na medium) and immediately frozen in liquid nitrogen. Na-dependent Ca-fluxes were measured using the Millipore filtration technique. Na-loaded sarcolemmal vesicles were diluted 200-fold in an isotonic K medium composed of 160 mM KCl, 20 mM Hepes, pH 7.4, and 20 µM $CaCl_2$, supplemented with 0.5 µCi ^{45}Ca/ml. Ca-uptake was determined at various intervals by filtering 100 µl aliquots through 0.22 um Millipore filters. The filters were rapidly rinsed with 4 ml of ice-cold 160 mM KCl, 20 mM Hepes, pH 7.4, and 0.5 mM $LaCl_3$ and radioactivity determined. When required, the Na-loaded sarcolemmal membranes were preincubated for 15 min with endothelin at various concentrations. The same concentration of endothelin was also added to the dilution medium (K-medium).

All drugs used were provided by the Chemistry Department of CIBA-GEIGY.

RESULTS

Endothelin, added cumulatively to the organ-bath at intervals of 15 min, produced a concentration-dependent increase in the tension of intact preparations of rat aorta (ED_{50}=0.8 nM) that was only marginally inhibited by the calcium-entry blockers nitrendipine, verapamil, and diltiazem (see Figure 1). The same concentrations of the calcium-entry blockers effectively inhibited contractions induced by potassium chloride (not shown). The potassium-channel opener, chromakalim, exerted an inhibitory effect similar to that of the calcium-entry blockers (Figure 1). Interestingly enough, endothelin-induced contractions were totally blocked by dichlorobenzamil but not by the same concentrations of amiloride (see Figure 1). Among the known amiloride

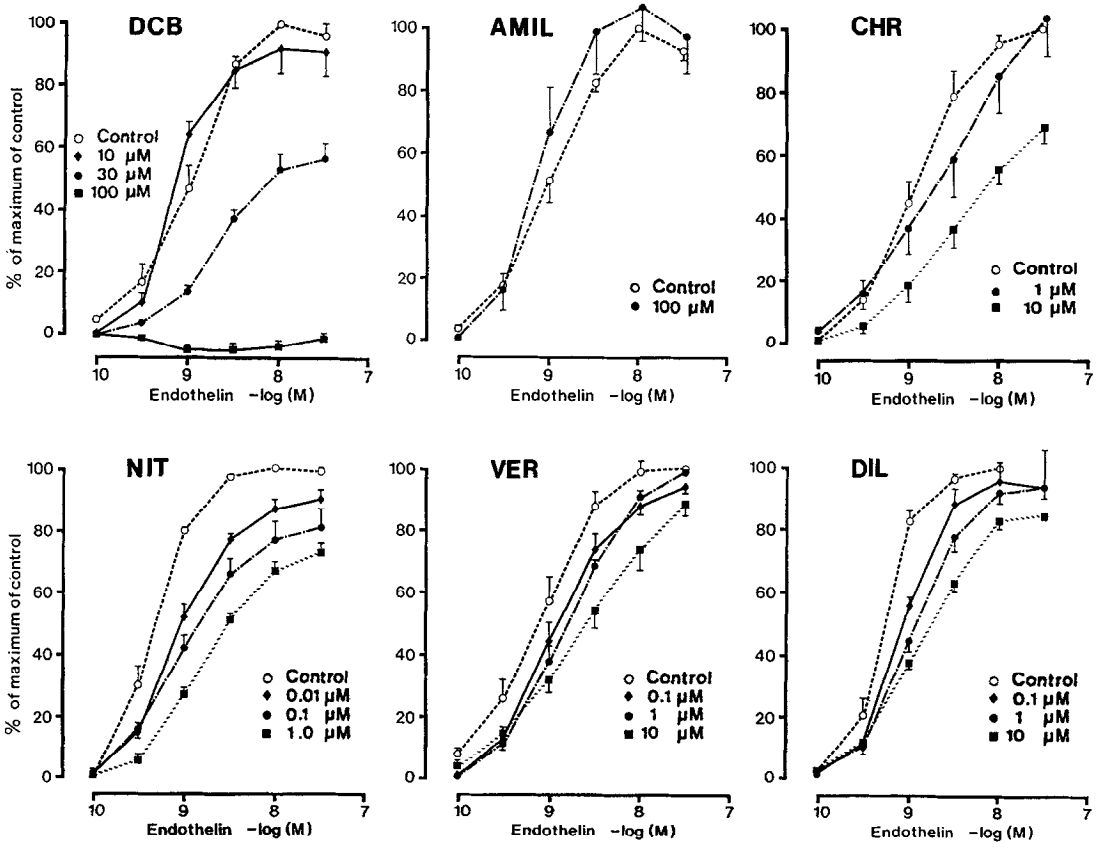

Figure 1
Effect of calcium-entry blockers, chromakalim, DCB and amiloride on endothelin-induced contraction in intact rat aortic rings. Concentration-effect curves were obtained in the absence and presence of dichlorobenzamil (DCB), amiloride (AMIL), chromakalim (CHR), nitrendipine (NIT), verapamil (VER) and diltiazem (DIL) as described in the Methods section. Responses are expressed as a percentage of the maximal response produced by endothelin in controls. Each curve is the mean of at least 4-6 observations.

derivatives, DCB is the most potent inhibitor of the sarcolemmal Na-Ca exchanger (IC_{50} 20 μM, see Ref.8). Therefore, the possibility that endothelin activates Ca influx via stimulation of Na-Ca exchange activity was thoroughly investigated. Isolated, sealed sarcolemmal membranes were used to study Na-induced Ca fluxes. Na-loaded sarcolemmal vesicles (160 mM NaCl) were diluted in iso-osmolar, Na-free medium to create a transient Na gradient and thus induce Ca uptake via the Na-Ca exchange route (Figure 2A). Ca efflux from Ca-loaded vesicles was then activated by a sudden increase of the Na concentration in the medium. Concurrently, the original Na gradient was dissipated (Na_{in} = Na_{out} = 0.8 mM), so that the addition of Na induced the formation of a subsequent, but reversed Na gradient.This procedure permitted the determination of the maximal velocity of Na-dependent Ca fluxes in both directions (i.e. Ca uptake and Ca release) at saturating Na concentrations and of the affinity of the exchanger for Na ions (see Figure 2A). Endothelin concentrations up to 30 nM were found to have no effect on the kinetics of the Na-Ca exchanger.

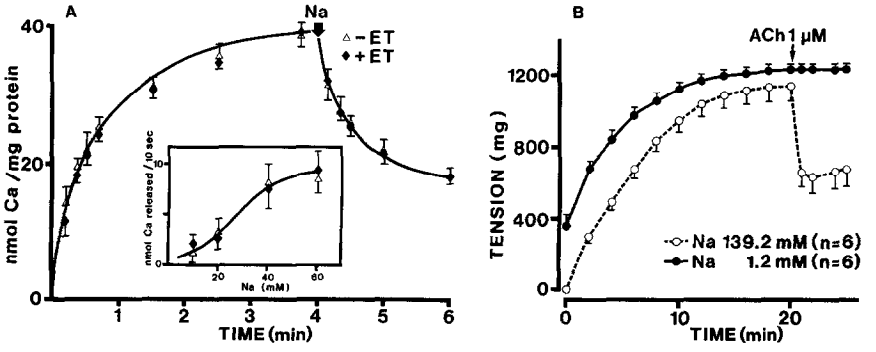

Figure 2A
Effect of endothelin on the Na-Ca exchanger. Ca uptake by sarcolemmal vesicles was determined as described in the Methods section. Na-loaded vesicles were diluted in a K medium to induce uptake. After 4 min (Na_{in}=Na_{out}=0.8 mM), 40 mM Na was added to the medium to induce Ca release via the exchange pathway. Insert: The rate of Ca release (Ca released during the first 10 sec) was determined as a function of the extravesicular Na concentration, which was varied from 10 to 60 mM. When required, 30 nM endothelin was added to the Na-loaded vesicles and to the dilution medium.
2B
Effect of low-sodium solution on the contractile response induced by endothelin in intact rings of rat aorta. Time-course of the contraction induced by endothelin 30 nM in normal-Na and low-Na PSS. At time 0, the pretension level of preparations incubated in low-Na PSS is about 370 mg, indicating that replacement of NaCl by choline chloride induced an increase in baseline tension. At peak contraction, ACh 1 μM was added.
Each curve is the mean of 6 observations.

Endothelin might stimulate Na-Ca exchange indirectly, i.e. via a second messenger, or via stimulation of the Na-H exchanger or of the Na-channels. To exclude these possibilities, the capacity of the hormone to induce contractions in Na-depleted aortic rings was investigated (see Figure 2B). After substitution of choline for Na ions in the organ-bath, a transient, outwardly oriented Na gradient across the cell membranes was formed. This induced a weak contraction of intact aortic rings (see Figure 2B, time 0), as previously reported (9). The rings were extensively preincubated in low-Na medium (4 washes over 30 min) to deplete intracellular Na before endothelin was applied. Figure 2B shows that the hormone was still capable of inducing maximal contractions in low-Na medium. The failure of ACh to induce relaxation demonstrated thatthe Na-Ca exchanger was not operational after 4 washes in low Na-medium. (Ca influx into endothelial cells via the Na-Ca exchanger is known to play a key role in the production and/or release of EDRF by ACh (10,11)). The lack of effect of amiloride (Figure 1) also indicates that the Na channels and the Na-H exchanger are not involved in the contractions induced by endothelin.

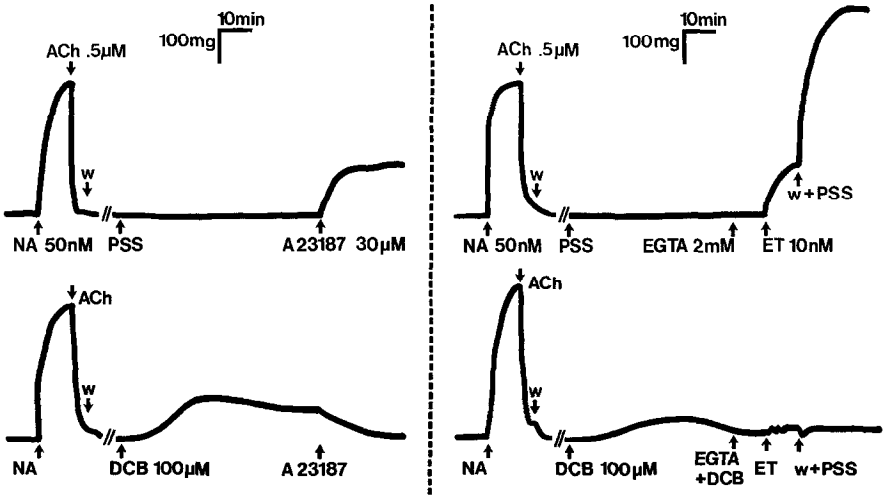

Figure 3
Typical tracings showing the effect of DCB on contractions induced by Ca influx via a Ca ionophore (A23187) (left panel) and by Ca release from intracellular pools (presence of EGTA, right panel). Note firstly the marked increase in tension after washing with physiological salt solution containing calcium (W+PSS) (endothelin is resistant to washing), and secondly the direct contractile effect of DCB. The relaxation of noradrenaline-induced (NA) contraction by acetylcholine (ACh) is indicative of the integrity of the endothelium. Tracings are representative of 4 experiments for each group.

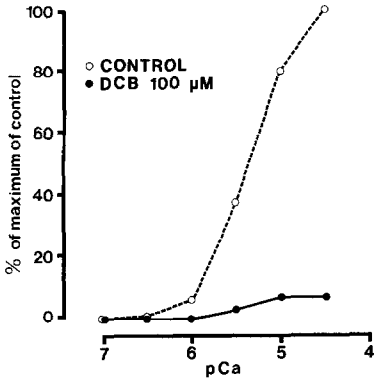

Figure 4
Effect of DCB on contractions induced by calcium in skinned (saponin-treated) aortic rings. Responses are expressed as percentage of the maximal response produced by calcium in the control. Each curve is the mean of 4 observations.

In the presence of normal Ca concentrations in the medium, the addition of a Ca ionophore to the aortic rings induces a slow influx of Ca ions into the smooth-muscle cells and hence a persistent contraction (12). In fact, Figure 3 shows that contractions could be elicited by the addition of 30 µM A23187. The potent inhibitory action of DCB on this contractile activity was also still fully in evidence. As shown in Figure 3, DCB at a concentration of 100 µM induced a slight increase in tension with a slow onset of action and a peak response between 15 and 30 min. This effect is compatible with its inhibitory action on Na-Ca exchange. After reaching its peak, the DCB-induced contraction slowly returned to initial values. In the presence of DCB, A23187 no longer induced contractions, but rather seemed to accelerate the return to baseline tension (Figure 3, left lower panel). This latter effect could be explained by the release of relaxing factor from the endothelial layer induced by the ionophore. Recent experiments with Indo-1-loaded smooth-muscle cells have shown that endothelin also induces a consistent elevation of IP_3 and of intracellular Ca levels after complexation of external Ca by EGTA (3). We further observed that endothelin was still capable of inducing consistent contractions in the absence of extracellular Ca (Figure 3, right upper panel). This contractile activity, which was solely dependent on the mobilization of Ca from intracellular pools, was also completely blocked by DCB. In the saponin-treated aortic rings, calcium induced a concentration-dependent contraction, which was almost completely blocked by DCB (Figure 4).

DISCUSSION

Studies in intact vessels have shown that endothelin-induced tension developments depend mainly on Ca entry into the smooth-muscle cells. Several routes of Ca entry are known (e.g. via the Na-Ca exchanger, Ca-channels) that could be activated by the hormone. In this study, we found that the amiloride-derivative DCB can completely inhibit endothelin-induced contractions. In a similar potency range, DCB is known to inhibit Na-dependent Ca fluxes (8), suggesting that stimulation of the exchanger could account for the contractile effects of endothelin. Our results, however, clearly demonstrate that Ca influx via the Na-Ca exchanger does not play a significant role in endothelin-induced contraction (see Figure 2 A and B). Endothelin was found to evoke maximal contractions even in Na-depleted vessels. This observation, together with the lack of effect of amiloride (100 μM), indicates that the Na channelsand the Na-H exchanger are also not involved in the action of endothelin, in accordance with a recent report (13). Recently, endothelin was reported to promote the opening of a new type of non-selective cation channel (3). Activation of these channels is supposedly very important, since, in addition to providing an independent pathway for Ca entry into the cell, it induces sustained depolarization of the membrane, thus also activating the voltage-dependent Ca channels (L-type). A selective Ca-channel blocker, such as diltiazem, verapamil or nifedipine, can only partially inhibit endothelin-induced contractions (see Figure 1 and Ref.3) since it does not prevent Ca entry via the non-selective cation channels. Similarly, a K-channel opener, such as chromakalim, hyperpolarizes the membrane, thus blocking the activation of the L-type Ca channels, but it does not interfere with the non-selective cation channels (see Figure 1). On the other hand, a blocker of the non-selective cation-channels would induce a more complete inhibition of endothelin-induced contractions, since both pathways for Ca entry are blocked (inhibition of the non-selective channels also prevents depolarization of the membrane and consequent opening of the voltage-dependent Ca channels). An interesting possibility is that DCB might block these non-specific cation channels. However, prolonged preincubation (more than 30min) is needed to evoke the potent inhibitory action of DCB, suggesting that its

target is located intracellularly, rather than superficially on the cell membrane. In addition, DCB was also found to induce powerful inhibition of the contractile activity when the excitatory Ca ions originate from an intracellular source (see Figure 3, right panel). It therefore seems that DCB interferes very late in the cascade of events leading from endothelin binding to contraction. The inhibitor can actually interfere directly with the activation of the myofilaments by Ca ions (Figure 3 and 4). Thus, DCB is potentially a highly versatile vasodilator capable of interfering with most types of vasocontrictor.

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