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Vasorelaxant properties of brefeldin A in rat aorta

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Abstract

The effects of brefeldin A, a putative specific agent that disassembles the Golgi apparatus were assessed on the contractility of de-endothelised rat aorta. Brefeldin A inhibited, either as pre- or as post-treatment, the contractions elicited by K^+ (75 mM) or phenylephrine (10 μM), being significantly more potent upon the latter. The thapsigargin (1 μM)-induced rat aorta contraction was less sensitive to brefeldin A inhibition. Pre-treatment with brefeldin A (30–100 μM) did not affect phenylephrine-induced transient contractions in Ca^{2+}-free medium, but strongly inhibited the phenylephrine-induced sustained contractions upon re-admission of Ca^{2+} to the medium. Brefeldin A was unable to prevent Ca^{2+} stores refilling. We concluded that brefeldin A inhibits Ca^{2+} entry but not the pathways activated after Ca^{2+} stores depletion or the pathways responsible for replenishment of these stores in rat aorta, presumably by disassembling the Golgi apparatus network. © 1997 Elsevier Science B.V.

Keywords: Brefeldin A; Smooth muscle, vascular; Contraction; Ca^{2+} entry

1. Introduction

Increases in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) are essential for almost any fundamental cellular process. These Ca^{2+} increases are the consequence of Ca^{2+} release from intracellular Ca^{2+} stores and of Ca^{2+} influx through more or less specific plasmalemmal ion channels (Berridge, 1995). These complex processes are under continuous debate. The precise localisation of functional intracellular Ca^{2+} stores is yet to be detailed. Pioneering work from the Somlyo group showed that, during smooth muscle stimulation, Ca^{2+} is mobilised from endo(sarco)plasmic reticulum, whereas mitochondria become functional only with higher (pathological) cytosolic Ca^{2+} concentrations (Somlyo et al., 1985). More recently, other 'candidates' were proposed as functional intracellular Ca^{2+} stores. The so-called 'calciosomes' were structurally identified in non-muscle cells (Volpe et al., 1990), while the nucleus has been shown to contain active Ca^{2+} stores, dischargeable in physiological conditions (Himpens et al., 1992). Moreover, it was demonstrated that the Golgi apparatus is clearly involved in intracellular Ca^{2+} regulation, through Ca^{2+} sequestration (Van Corven et al., 1986; Mughal et al., 1989) and Ca^{2+} release in response to inositol (1,4,5)-trisphosphate (IP_3, Yoshimoto et al., 1990; Zha et al., 1995), or to Ca^{2+} ionophores (Chandra et al., 1990).

In addition to Ca^{2+} release from intracellular stores, Ca^{2+} entry is essential for most sustained cellular responses (Berridge, 1995). In non-excitable cells, Ca^{2+} influx may be operated (i.e. determined and regulated) by (a) the filling state of Ca^{2+} stores; (b) membrane receptors directly; (c) second messengers such as inositol (1,3,4,5) tetrakisphosphate (IP_4), IP_3 or Ca^{2+} and (d) any conjectural association between the aforementioned. In excitable tissues, such as smooth muscle, there is in addition to these pathways, a major contribution of voltage-dependent Ca^{2+} entry (Felder et al., 1994).

In rat aortic smooth muscle, as in most other smooth muscles, contractions are accompanied by $[Ca^{2+}]_i$ increases (Rico et al., 1990). This can be obtained either after agonist stimulation or in the absence of receptor activation. Agonists such as phenylephrine, serotonin or angiotensin II, by interacting with specific receptors, induce both Ca^{2+} release and Ca^{2+} entry through receptor-operated Ca^{2+} channels; in the rat aorta a contribution of voltage-operated Ca^{2+} channels cannot be excluded (Rico

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et al., 1990). Elevation of extracellular K^+ concentration produces Ca^{2+} entry through voltage-operated Ca^{2+} channels (Marriott, 1988). Agents which deplete intracellular Ca^{2+} stores without membrane receptor activation (e.g. thapsigargin) evoke Ca^{2+} increases related to both receptor-operated Ca^{2+} channels and voltage-operated Ca^{2+} channels (Low et al., 1993).

Brefeldin A is a macrolytic cytotoxic lactone that has been shown to disassemble the Golgi apparatus network (Betina, 1992). Its effect is the consequence of blocking the association of a 110 kDa protein with the Golgi apparatus (Donaldson et al., 1990). The purpose of the present work was to investigate the effects of brefeldin A in the isolated rat aorta. Brefeldin A inhibited the contractions evoked by phenylephrine rather than those induced by high K^+ or that induced by thapsigargin. Moreover, brefeldin A selectively inhibited the Ca^{2+} entry dependent part of the phenylephrine-induced contractions.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats weighing 150–200 g (12–14 weeks old) were killed by cervical dislocation and exsanguinated. The descending thoracic aorta was carefully removed and immersed in modified Krebs Henseleit solution (physiological salt solution, PSS) with the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.6; $NaHCO_3$, 24.9; EDTA, 0.03; glucose, 5.55 (pH 7.3 adjusted with NaOH). The aorta was dissected free of the surrounding connective tissue and cut into 2 mm wide segments. The endothelium was removed by gentle rubbing with a smooth softwood stick. Each ring was mounted vertically between a pair of stainless steel wire hooks in 2 ml organ baths containing PSS warmed at 37°C and continuously aerated with 95% O_2 and 5% CO_2. The upper hook was attached to an isometric force transducer type IMF 002. The electrical signal was converted to digital data by a Kaethley A/D 2000 board and recorded with a PC 486, using a data acquisition program (PeakExe).

2.2. Experimental procedures

A pre-tension of 20 mN (2 g) was imposed on each preparation; this ensured maximal contractile responses on pharmaco–mechanical stimulation. After an equilibration period of 60–90 min, the arteries were stimulated 2–3 times with 10 μM phenylephrine until at least two successive contractions differing by less than 5% were obtained. At the plateau of one of these contractions the efficient removal of endothelium was tested by the absence of relaxation in response to 10 μM carbachol. The amplitude of the last phenylephrine 10 μM-induced contraction was considered as a control (100%) for further comparisons. Rings which did not develop 0.8 g active force in response to phenylephrine were discarded.

Post-treatment dose effect curves were obtained after the contractions reached a plateau and maintained it for at least 15 min with variations less than 2% from the total contractile force developed. Brefeldin A (3–100 μM) was added in a cumulative manner at 15 min intervals after a stable plateau was reached with the last concentration. Pre-treatment protocols initially implied a variable time incubation with the desired concentration of brefeldin A and thereafter addition of the contractile agent. After the time-dependence of brefeldin A effects were established (see Section 3 Fig. 1a), all subsequent experiments were performed with the optimal 15 min incubation period. Experiments assessing the contractile efficacy of phenylephrine in the absence of extracelullar Ca^{2+} were performed using 'Ca^{2+}-free' PSS (without $CaCl_2$ and with 2.5 mM EGTA). The arteries were washed 4 times with Ca^{2+}-free PSS and 10^{-5} M phenylephrine was added 5 min thereafter.

2.3. Drugs

Brefeldin A (γ, 4-dihydroxy-2-[6-hydroperoxy-1-heptenyl]-4 -cyclopentanoecrotonic acid λ-lactone), lot 51H4047, was purchased from Sigma (St. Louis, MO, USA). A stock solution, 100 mM, was prepared in absolute ethanol (96%) and stored at 4°C until used. The final concentration of ethanol in the bath was less than 0.1%, without having an effect on the contractile behaviour of rat aortic rings in the control experiments ($n = 4$).

Thapsigargin, lot 73H0860 was dissolved in ethanol (stock solution, 1 mM) and stored at −20°C. The final concentration of ethanol in the organ bath was identical to that used for brefeldin A. All other drugs were of research grade (Sigma) and were dissolved in bidistilled water on the day of the experiment as concentrated stock solutions.

2.4. Statistics

All series were performed in at least four different animals ($n \geq 6$). As detailed in Section 3 the maximal relaxant effect of brefeldin A did not even reach 100 μM. Because of this we did not calculate IC_{50} values. The unpaired Student's t-test was used with $P < 0.05$ being considered significantly different.

3. Results

For the pre-treatment protocols, we first tested the time dependence of brefeldin A effects. For this purpose, the preparations were incubated for a different period of times with an intermediate concentration of brefeldin A, 30 μM (see below). As shown in Fig. 1a, the inhibitory effect of brefeldin A on phenylephrine-induced contractions started

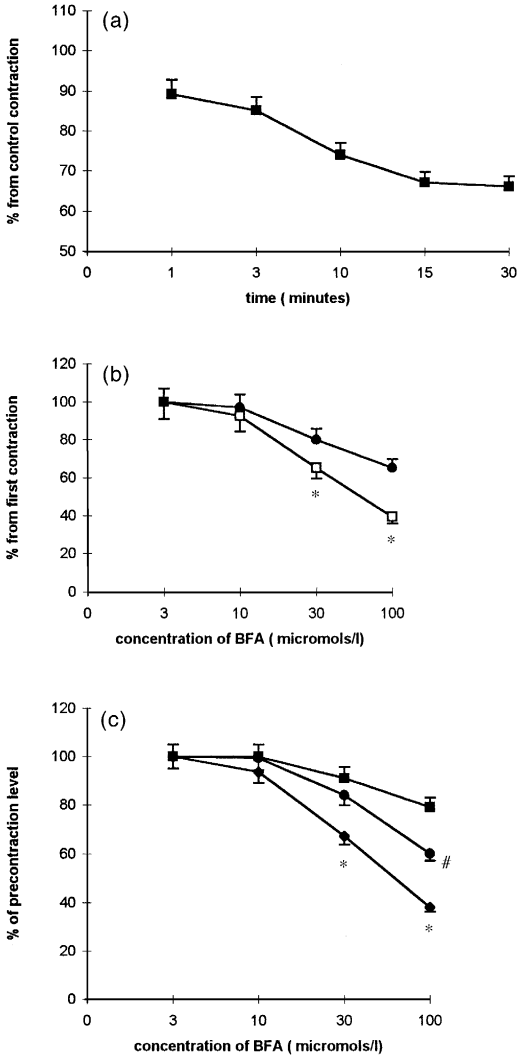

Fig. 1. (a) Time-dependence of the inhibitory effect of 30 μM brefeldin A on 10 μM phenylephrine-induced rat aorta contractions. Preparations were incubated with brefeldin A for the time shown on the horizontal axis and thereafter stimulated with phenylephrine. The amplitude of this response was compared to that of the previously obtained control response. ($n = 6$ in each case, mean ± S.E.M.). (b) Concentration–effect curves for the inhibitory effects of brefeldin A administered as pre-treatment on the 10 μM phenylephrine (□) or 75 mM KCl (●)-induced contractions ($n = 6$ in each case, mean ± S.E.M.). * Indicates statistically significant differences at $P < 0.05$. (c) Concentration–effect curves for brefeldin A relaxing effects upon the plateau of 10 μM phenylephrine-(♦), KCl 75 mM- (●) and 1 μM thapsigargin (■)-induced contractions of rat aorta rings ($n = 6$ in each case, mean ± S.E.M.). * Indicates statistically significant differences between phenylephrine and the two other contractile agents at $P < 0.05$. # Indicates statistically significant differences between KCl and thapsigargin, also at $P < 0.05$.

after 1 min, maximum relaxation being reached after more than 10 min. We therefore used 15 min as the incubation period for the pre-treatment experiments. Fig. 1b shows the concentration dependence of the relaxing action of brefeldin A, administered as pre-treatment, on the 75 mM K^+- and 10 μM phenylephrine-induced contractions.

Concentration–effect curves were made for the effects of brefeldin A on the contractions induced by 10 μM phenylephrine, 75 mM K^+ and 1 μM thapsigargin (Fig. 1c) in normal PSS. Under control conditions, the contractions induced by 75 mM K^+ and 10 μM thapsigargin were 87 and 47%, respectively, of the contractions induced by 1 μM phenylephrine ($n = 12$, $n = 6$, respectively). These concentrations determined the maximal contractile response for the respective agents, which was reached after approximately 15 min in the case of K^+-induced contractions and 30 min in the case of thapsigargin-induced contraction. Brefeldin A had relaxing effects on all the contractions mentioned, starting from 10 μM, the maximal effect not being reached at 100 μM. Higher brefeldin A concentrations (300 μM) fully relaxed all these types of contractions (data not shown). The IC_{50} values were not calculated, since with such a high concentration, they were probably not specific for a direct effect on the Golgi apparatus (Betina, 1992); moreover, as the concentration of ethanol in the organ bath exceeded 0.3% (v/v), there were alterations in the contractile behaviour of rat aortic rings (data not shown).

The apparent potency order for brefeldin A relaxant effects was phenylephrine > KCl > thapsigargin; this was similar to that in the pre-treatment experiments (phenylephrine > KCl). There were no statistically significant differences between the relaxing effects of brefeldin A whether administered as post- or as pre-treatment for both phenylephrine- and KCl-induced contractions (Fig. 1b and c).

In the absence of extracellular Ca^{2+}, 10 μM phenylephrine induced a transient contraction of 29 ± 4% ($n = 12$, Fig. 2a) of the control. Further addition of phenylephrine or thapsigargin did not elicit any contractile response, indicating that under these circumstances phenylephrine ensured maximal depletion of Ca^{2+} stores. Readmission of Ca^{2+} to restore the initial 2.5 mM concentration, in the continuous presence of phenylephrine, resulted in a slowly developing stable contraction of 118 ± 4.5% ($n = 6$, Fig. 2a). As shown in Fig. 2b and Fig. 3a, brefeldin A (either 30 μM or 100 μM) was unable to affect the phenylephrine-induced contraction in Ca^{2+}-free PSS. In contrast, brefeldin A (either 30 μM or 100 μM) strongly inhibited the contractions induced by readmission of $CaCl_2$ in the medium (Fig. 2b and c). At these concentrations brefeldin A was significantly more potent than on phenylephrine induced contractions in normal PSS, in either pre- or post-treatment protocols (41 ± 2% versus 62 ± 4% in pre-treatment and 65 ± 5% in post-treatment for 30 μM brefeldin A, $n = 6$ in each case, $P = 0.021$ and $P = 0.017$, respectively).

We also investigated whether brefeldin A could affect the spontaneous refilling of the phenylephrine-sensitive intracellular Ca^{2+} stores following depletion. The protocol used was described previously (Bourreau et al., 1993; Filipeanu et al., 1995). After the transient phenylephrine contraction in Ca^{2+}-free PSS, the arteries were repeatedly

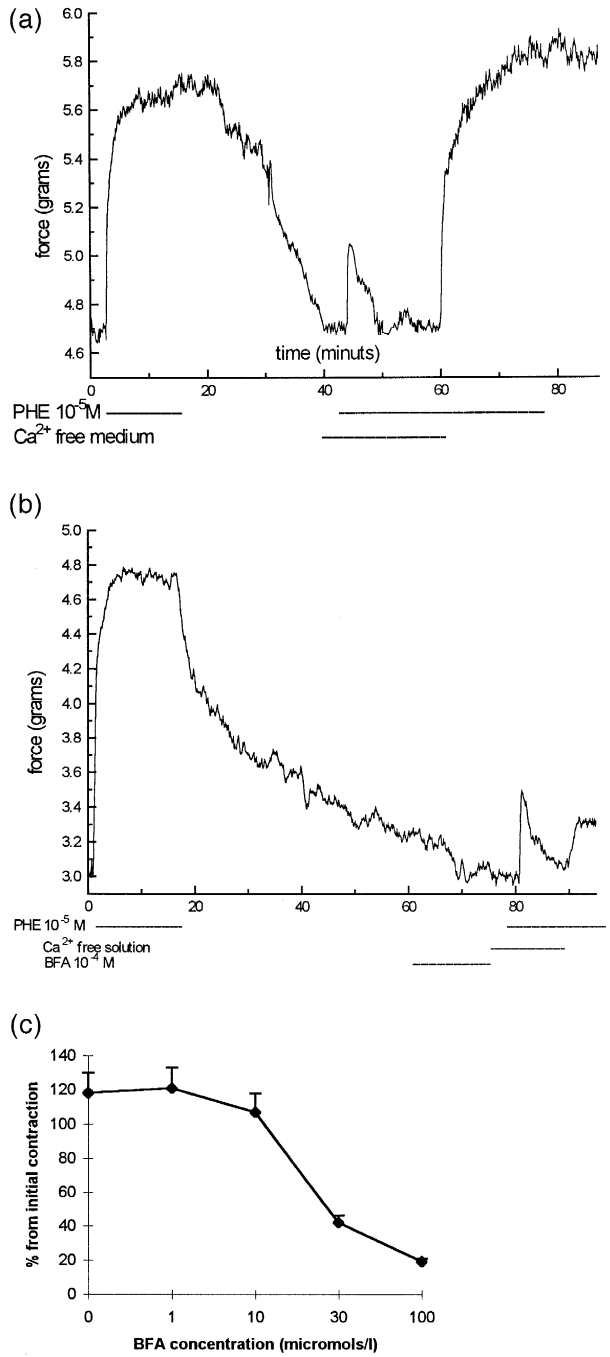

Fig. 2. (a) Original trace showing effects of 10 μM phenylephrine in Ca^{2+}-free medium and after restoration of normal Ca^{2+} concentration in the bath. (b) Original trace showing that 100 μM brefeldin A did not affect the transient contractile response to 10 μM phenylephrine in Ca^{2+}-free medium, but strongly inhibited the sustained contractile response obtained on readmission of Ca^{2+} in the medium in continuous presence of phenylephrine. (c) Concentration–effect curve for brefeldin A on the sustained response induced by 10 μM phenylephrine at readmission of Ca^{2+}. Comparisons with control contractions with 10 μM phenylephrine at the beginning of experiments are expressed as % on the vertical axis ($n = 6$ in each case, mean ± S.E.M).

washed with Ca^{2+}-free PSS to ensure the complete removal of the agonist. The medium was changed to normal PSS for 15 min, and the Ca^{2+}-free protocol was then repeated. The 10 μM phenylephrine stimulation was performed again and the amplitude of this response was compared to the first one. None of the concentrations of brefeldin A (present only during the 'refilling period') affected the second phenylephrine-induced transient contractions in Ca^{2+}-free PSS (Fig. 3a and b). Caffeine did not induce a contractile response in rat aorta at 37°C in Ca^{2+}-free PSS (Noguera and D'Ocon, 1992; Filipeanu et al., 1995). For this reason we did not test the effects of brefeldin A on refilling of the caffeine sensitive intracellular Ca^{2+} stores.

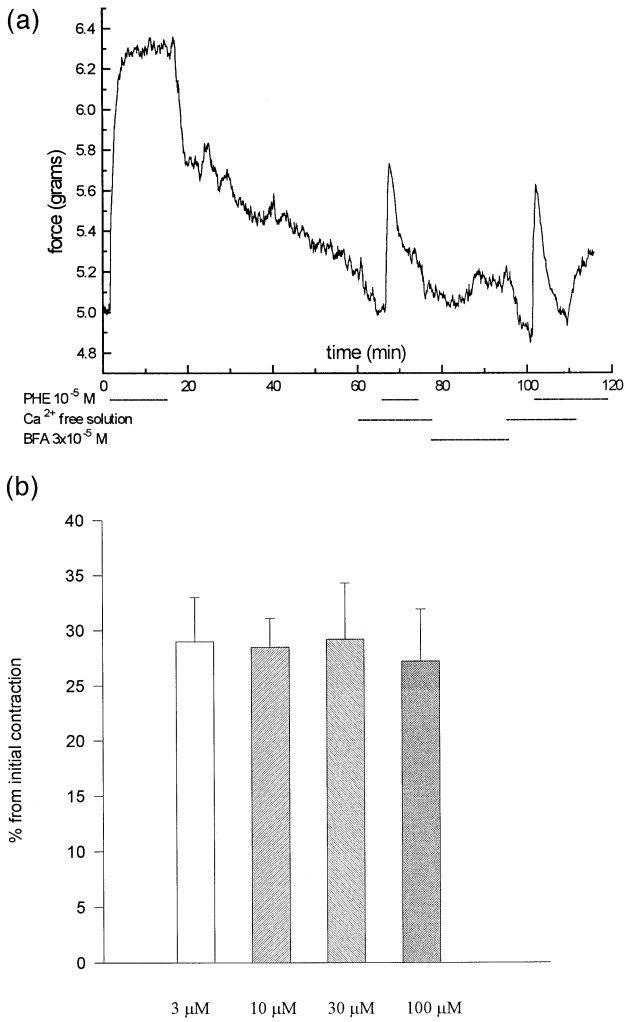

Fig. 3. (a) Original trace illustrating the lack of effect of brefeldin A on intracellular Ca^{2+} stores refilling in de-endothelised rat aorta. After the control contractions the preparations were immersed in Ca^{2+}-free solution with 2.5 mM EGTA and 5 min later were stimulated with 10 μM phenylephrine. After the transient contractions returned to their basal levels, the preparations were extensively washed with Ca^{2+}-free solution to ensure removal of the agonist. For the 15 min following, the preparations were exposed to normal PSS (2.5 mM Ca^{2+}) plus different concentrations of brefeldin A. Thereafter the rate of filling of intracellular Ca^{2+} stores was tested again as the response to 10 μM phenylephrine. This trace is one representative of six obtained using 30 μM brefeldin A. (b) The second panel shows the of brefeldin A effects on refilling of phenylephrine-sensitive intracellular Ca^{2+} stores assessed as described for the first panel ($n = 6$ in each case, mean ± S.E.M.).

4. Discussion

Our results demonstrated that brefeldin A was able to inhibit rat aorta contractions evoked by either the α_1 agonist, phenylephrine, or KCl. To a lesser extent, brefeldin A also inhibited the contractions evoked by the sarco(endo)plasmic Ca^{2+} ATP-ase inhibitor, thapsigargin.

Brefeldin A is a macrolytic lactone (Betina, 1992) which disassembles the Golgi apparatus after interaction with a 110 kDa protein (Donaldson et al., 1990). To our knowledge no other effects of brefeldin A have been reported so far. The impairment of intracellular Ca^{2+} homeostasis by brefeldin A has been shown in LLC-PK_1 cells (Zha et al., 1995) and in neuroblastoma cells (Passafaro et al., 1994). Moreover a role for the Golgi apparatus in sequestration of Ca^{2+} (Chandra et al., 1990) and in Ca^{2+} release in response to IP_3 (Chandra et al., 1990; Yoshimoto et al., 1990; Zha et al., 1995) has been demonstrated. The concentrations required and the time dependence of brefeldin A relaxing effects in this study were close to the ones effective on Golgi apparatus in LLC-PK_1 cells (Zha et al., 1995).

The fact that brefeldin A did not affect the transient contractions induced by phenylephrine in Ca^{2+} free medium suggests that, in rat aorta, the Golgi apparatus is not an adrenergic-sensitive Ca^{2+} store involved in contraction. This finding differs from the situation in epithelial cells (Chandra et al., 1990; Zha et al., 1995) and indicates that the role of the Golgi apparatus could be different according to the cell type. This finding also demonstrates that less than 100 μM brefeldin A did not impair the phenylephrine-adrenoreceptor coupling in rat aorta. In addition to releasing Ca^{2+} from the endoplasmic reticulum, adrenergic activation of aortic smooth muscle determines Ca^{2+} entry from extracellular space; this influx is essential for the sustained phenylephrine-induced contraction (Rico et al., 1990). While brefeldin A was ineffective on the contraction induced by phenylephrine in Ca^{2+}-free PSS, it inhibited the contraction upon re-addition of Ca^{2+} in the continuous presence of the agonist. Moreover, the brefeldin A relaxing effect was significantly greater under these circumstances than observed in pre- or post-treatment protocols. These data show that brefeldin A alters the phenylephrine-induced contraction mainly by impairing Ca^{2+} entry from the extracellular space.

Phenylephrine activates both voltage-independent and voltage-dependent Ca^{2+} entry in rat aorta (Rico et al., 1990; Marriott, 1988). Brefeldin A relaxing effects were seen with either pre- or post-treatment protocols, on both K^+- and phenylephrine-induced rat aorta contractions. K^+-induced rat aorta contractions are mainly related to activation of voltage-operated Ca^{2+} entry (Marriott, 1988). We can conclude from these results that brefeldin A may alter voltage-operated Ca^{2+} entry, possibly by modulating translocation of these channels to the plasma membrane, as has been shown in neuroblastoma cells (Passafaro et al., 1994). However, the brefeldin A inhibitory effects on phenylephrine-induced rat aorta contractions were significantly greater than those on K^+-induced contractions in both pre- and post-treatment protocols. Thus in parallel with the inhibition of voltage-operated Ca^{2+} channels, brefeldin A may also influence voltage-independent Ca^{2+} entry in rat aorta. According to a recent classification of voltage-independent Ca^{2+} channels, these can be divided into channels opened by the emptying of intracellular Ca^{2+} stores, or by second messengers and/or by direct interaction of an agonist with its receptor (Felder et al., 1994). Of the three contractile agents used in this study, thapsigargin induced a rat aorta contraction that was least sensitive to brefeldin A relaxation. Thapsigargin induces contractions of the rat aorta by promoting extracellular Ca^{2+} entry as a consequence of discharging intracellular Ca^{2+} stores (Low et al., 1993). Taking into account that brefeldin A effects were minimal on thapsigargin-induced rat aorta contractions and on spontaneous Ca^{2+} stores refilling we concluded that brefeldin A does not affect the Ca^{2+} entry putatively subsequent to emptying of intracellular Ca^{2+} stores.

The exact site of brefeldin A action in rat aorta contractions remains to be determined. The relaxing effects may be related to the new hypothesis regarding Ca^{2+} entry and a tubule like connections with the extracellular space (Bode and Netter, 1996). Brefeldin A can affect the organization of microtubules and can also impair the release and trafficking of small GTP-binding proteins (Roa et al., 1993), which are involved in regulation of Ca^{2+} entry (Bode and Netter, 1996; Bird and Putney, 1993) and smooth muscle contractile events (Gong et al., 1996). Inhibition of Na^+ channels by brefeldin A (Fisher et al., 1996) can also explain some of the relaxing effects observed in the present study.

In conclusion, the present work showed that the macrolytic antibiotic, brefeldin A, is able to relax rat aorta contractions, being most potent upon contractions evoked by α_1-adrenergic receptor stimulation, by inhibition of Ca^{2+} entry probably related to disruption of the Golgi apparatus.

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