

## On the mechanisms of the antispasmodic action of some hindered phenols in rat aorta rings

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### Abstract

The antispasmodic effects of 3-*t*-butyl-4-hydroxyanisole (BHA) and some structurally related compounds were investigated in endothelium-intact rat aorta rings. Nordihydroguaiaric acid (NDGA), BHA, 3,5-di-*t*-butyl-4-hydroxyanisole (DTBHA), 2,6-di-isopropyl phenol (propofol) and 2,2'-dihydroxy-3,3'-di-*t*-butyl-5,5'-dimethoxydiphenyl (DIBHA) did not cause relaxation when added at the plateau of phenylephrine-evoked contraction, nor did they affect the concentration–relaxation curve for acetylcholine in precontracted rings. In rings depolarised with physiological salt solution (PSS) containing 40 mM K<sup>+</sup>, NDGA, BHA, DTBHA, 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ), propofol and nifedipine, but not DIBHA, inhibited the contraction induced by cumulative addition of Ca<sup>2+</sup> (0.05–10 mM) in a concentration-dependent manner; this inhibition was inversely related to the Ca<sup>2+</sup> concentration. In 40 mM K<sup>+</sup> PSS, 25 nM nifedipine blocked the 1 mM Ca<sup>2+</sup>-induced contraction, whereas 50  $\mu$ M DTBHA, NDGA, BHA, BHQ and propofol significantly antagonised it by 84.4%, 73.0%, 52.8%, 45.6% and 35.7%, respectively. In the presence of 1  $\mu$ M methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644), the response to Ca<sup>2+</sup> did not differ from control values with nifedipine and BHQ, was partially restored with DTBHA and NDGA, and was not affected with BHA and propofol. Nifedipine markedly inhibited (85.2%) the Ba<sup>2+</sup>-induced contraction and this effect was totally reversed by Bay K 8644. BHA and DTBHA showed antispasmodic activity (45.3% and 43.1%, respectively) which was partly reversed by Bay K 8644. In contrast, Bay K 8644 did not affect the inhibition exerted by BHQ, NDGA and propofol (69.5%, 53.3% and 46.1%, respectively). Nifedipine, BHA, DTBHA, propofol and NDGA inhibited the contractile response to 1 mM Ca<sup>2+</sup> of aorta rings depolarised with 40 or 80 mM K<sup>+</sup> PSS to a similar extent. Cromakalim inhibited the Ca<sup>2+</sup>-evoked contraction only in 30 mM K<sup>+</sup> PSS and BHQ only in 80 mM K<sup>+</sup> PSS. DIBHA had no effect on this model. Cromakalim, but not BHA, stimulated <sup>86</sup>Rb<sup>+</sup> efflux from ring preparations. In 80 mM K<sup>+</sup> PSS containing 1  $\mu$ M nifedipine, only papaverine affected the phenylephrine-induced contraction. Moreover, when the rings were preincubated with 1 mM Ni<sup>2+</sup>, the response to phenylephrine in the presence of BHQ was significantly reduced. In conclusion, we propose that BHA may non-specifically inhibit Ca<sup>2+</sup> influx at the plasmalemma level rather than affect the function of K<sup>+</sup> channels, Ca<sup>2+</sup> release from intracellular stores or endothelium-dependent relaxation. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Hindered phenol; BHA (3-*t*-butyl-4-hydroxyanisole); Ca<sup>2+</sup> homeostasis

### 1. Introduction

Previous studies from this laboratory have shown that, besides their well-known antioxidant activity, a series of phenols that are structurally related to 3-*t*-butyl-4-hydroxyanisole (BHA) have antispasmodic and spasmolytic activ-

ity in rat ileum longitudinal muscle (Sgaragli et al., 1993b) and guinea pig gastric fundus preparations (Fusi et al., 1998b). Compounds with such dual pharmacological activities are particularly valuable because they block the two most crucial steps in the sequence of events triggered by ischemia–reperfusion injury: free radical release and Ca<sup>2+</sup> overload.

Ca<sup>2+</sup> homeostasis has been extensively studied in rat aorta rings (Himpens et al., 1995; Karaki et al., 1997). The increase in cytoplasmic Ca<sup>2+</sup> concentrations which triggers the contractile process in vascular smooth muscle cells may be due to increased permeability of the cell

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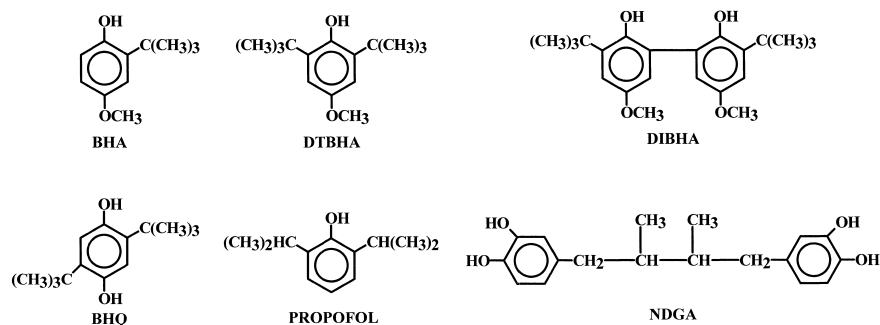


Fig. 1. Molecular structures of the compounds studied.

membrane to extracellular  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  entry) or to mobilisation of  $\text{Ca}^{2+}$  from cellular stores. The contractile responses are differentially susceptible to vasodilators acting through various mechanisms (Karaki, 1987). A first screening of drugs affecting vascular smooth muscle contraction can be performed by analysing their effects on the contractile responses of isolated rat aorta.

Preliminary data indicate that BHA is an active substance which also has antispasmodic effects on vascular smooth muscle (Gorelli et al., 1995; Fusi et al., 1999). The present study was undertaken to obtain a deeper insight into the effects of BHA on  $\text{Ca}^{2+}$  movements in smooth muscle and to elucidate its mechanism of action. This was done by comparative analysis of some phenol derivatives structurally related to BHA (Fig. 1).

## 2. Materials and methods

### 2.1. Aorta ring preparation and equilibration period

Male Wistar rats (250–350 g) were anaesthetised with a mixture of Ketavet® (Gellini, Italy) and Rompum® (Bayer, Germany), decapitated and bled. The thoracic aorta was immediately removed, cleaned of adhering fat and connective tissue and cut into 1.5-mm wide rings with a razor blade slicing device. Care was taken to avoid abrasion of the intimal surface of the rings in order to maintain the integrity of the endothelial layer. Each arterial ring was mounted over two rigid parallel stainless steel tubes, one fixed in place and the other attached to an isometric transducer (Basile, Varese, Italy). The preparation was immersed in a water-jacketed organ bath (37°C) containing 5 ml of a modified Krebs–Henseleit physiological salt solution (PSS) (composition mM: NaCl 124, KCl 4,  $\text{CaCl}_2$  1.8,  $\text{MgCl}_2$  1.1,  $\text{KH}_2\text{PO}_4$  0.4,  $\text{NaHCO}_3$  25 and glucose 5.5) bubbled with a 95%  $\text{O}_2$ –5%  $\text{CO}_2$  gas mixture to give a pH of 7.4. PSS containing 80 mM KCl was prepared by replacing NaCl with equimolar KCl. The vessel segments were allowed to equilibrate for 1 h at a resting tension of 1 g. During the equilibration period, PSS was changed every 15 min. After the equilibration period, the aorta rings were stimulated with 80 mM  $\text{K}^+$  PSS until a sustained response

was obtained ( $\sim 15$  min), in order to test their contractile capacity. Under these conditions, maximal plateau values of active tension of about 339.4 mg were obtained. Following a 30-min washout, the presence of functional endothelium was assessed in all preparations by testing the capacity of acetylcholine (10  $\mu\text{M}$ ) to induce relaxation of rings precontracted with phenylephrine (Furchgott and Zawadzki, 1980). The rings were then washed and equilibrated for another 30–60 min before testing the different experimental settings (see below). Control preparations were treated with vehicle only.

### 2.2. Endothelium-dependent relaxation

In endothelium-intact aorta rings, a cumulative concentration–contraction curve for phenylephrine (1 nM–30  $\mu\text{M}$ ) was recorded in order to determine the  $\text{ED}_{90}$ . The rings were then washed for 30–45 min, changing PSS every 15 min. When baseline tension was restored, contraction was provoked by phenylephrine ( $\text{ED}_{90}$ ) and a cumulative concentration–relaxation curve for acetylcholine was obtained. A second washing (45 min) followed. The contraction procedure was repeated and a second cumulative concentration–relaxation curve for acetylcholine was obtained in the presence of the test compounds, which were added once a plateau level was reached. At the end of each experiment, 10  $\mu\text{M}$  acetylcholine followed by 10  $\mu\text{M}$  sodium nitroprusside was added to test muscle functional integrity.

### 2.3. Concentration–response curve for $\text{Ca}^{2+}$

The antispasmodic response to  $\text{Ca}^{2+}$  (0.05–10 mM) was studied by obtaining cumulative concentration–response curves for rings depolarised with  $\text{Ca}^{2+}$ -free 40 mM  $\text{K}^+$  PSS. The test substances were present for 15 min before, as well as throughout the concentration–response curve procedure.

### 2.4. $\text{Ca}^{2+}$ - and $\text{Ba}^{2+}$ -induced contractions

In a first series of experiments, the basal contractile response of the preparations was tested by measuring

muscle contraction in response to 1 mM  $\text{Ca}^{2+}$  added to  $\text{Ca}^{2+}$ -free 40 mM  $\text{K}^+$  PSS, for a fixed period of 30 min. This period was sufficient to reach an optimum contraction plateau. Repeatability of this contraction was proved by a second application of  $\text{Ca}^{2+}$  (control). Washing with a  $\text{Ca}^{2+}$ -free PSS every 15 min for a period of 45 min was used to avoid tachyphylaxis. The test substances were then added 15 min before the third application of  $\text{Ca}^{2+}$  to evaluate their antispasmodic activity.

In a second series of experiments, 5 mM  $\text{Ba}^{2+}$  was used as contractile agent. The experiments were carried out in  $\text{Ca}^{2+}$ - and phosphate-free PSS to avoid precipitation. The test compounds were added 15 min before  $\text{Ba}^{2+}$ .

Both experimental protocols (i.e.  $\text{Ca}^{2+}$ - and  $\text{Ba}^{2+}$ -induced contractions) were repeated in the presence of 1  $\mu\text{M}$  methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644). The effect of the compounds was evaluated as a percentage of control.

## 2.5. Assessment of $\text{K}^+$ channel opening activity

The antispasmodic activity of the test drugs was evaluated under different conditions of high  $\text{K}^+$ -evoked depolarization (30 or 80 mM  $\text{K}^+$ ) and compared to the activity of nifedipine or cromakalim (Gurney, 1994). The compounds were tested against 1 mM  $\text{Ca}^{2+}$ -induced contraction in the presence of 30 or 80 mM  $\text{K}^+$ .

In another series of experiments, the effect of BHA on  $\text{K}^+$  fluxes was tested by radioassay of  $^{86}\text{Rb}^+$  efflux, used as an adequate marker of  $\text{K}^+$  flux (Bolton and Clapp, 1984). Aorta rings were impaled on a syringe needle attached to a Perspex gassing pump. The preparations were immersed in a tube containing 3 ml normal PSS and were incubated for 30 min, gassed with 95%  $\text{O}_2$  + 5%  $\text{CO}_2$  mixture at 37°C. For loading with  $^{86}\text{Rb}^+$ , rings were incubated for an additional 120 min in PSS containing  $^{86}\text{Rb}^+$  5  $\mu\text{Ci}/\text{ml}$ . After loading, the rings were briefly dipped in PSS to remove excess radioactivity and then  $^{86}\text{Rb}^+$  was allowed to efflux from the tissue by transferring the rings to tubes containing 3 ml of 20 mM  $\text{K}^+$  PSS for 10 successive 2-min periods, established to be sufficient to obtain a stable  $^{86}\text{Rb}^+$  efflux. The tissue was then immersed in a tube containing 20 mM  $\text{K}^+$  PSS and the following substances were added: 100  $\mu\text{M}$  cromakalim for 10 min, 100  $\mu\text{M}$  BHA for 16 min or 14 mM dimethyl sulphoxide (DMSO) for 16 min. At the end of the efflux period, 1-ml aliquots of the solutions were added to 4 ml Ultima Gold (Packard) scintillation mixture and counted for radioactivity in the Cerenkov mode at 50% efficiency. The radioactivity remaining in the tissue at the end of the assay was determined by dissolving the tissue overnight at 50°C in 500  $\mu\text{l}$  1 N HCl and counting in the  $^{32}\text{P}$  channel at 100% efficiency. Calculations and evaluation of the data were done as described by Quast (1987).

## 2.6. Contraction induced by mobilization of $\text{Ca}^{2+}$ from intracellular stores

The contribution of intracellular  $\text{Ca}^{2+}$  stores to muscle contraction was determined as already described (Fusi et al., 1998a).

## 2.7. Drugs: commercial sources and solutions

The dimer of BHA, 2,2'-dihydroxy-3,3'-di-*t*-butyl-5,5'-dimethoxydiphenyl (DIBHA), was synthesized by direct oxidation of BHA as described elsewhere (Sgaragli et al., 1980). BHA, (from Merk-Schuchardt, Germany), 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ), 2,6-di-isopropyl phenol (propofol), nordihydroguaiaretic acid (NDGA), papaverine and cromakalim (all obtained from Sigma Chimica, Milan, Italy) and 3,5-di-*t*-butyl-4-hydroxyanisole (DTBHA) (from Aldrich-Chemie, Steinheim, Germany) were dissolved in 100% DMSO and shielded from light with aluminium foil; nifedipine and Bay K 8644 (Sigma) were dissolved in absolute ethanol. DMSO and ethanol did not exceed 0.1% (v/v) in the bath, at which concentrations they had no effect. Drug solutions were prepared daily. The concentrations of the agents are given as the final molar concentrations in the bath.

## 2.8. Statistical analysis

All values are expressed as means  $\pm$  S.E.M.;  $n$  is the number of rats (in brackets). One-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons and Student's *t*-test were performed using GraphPad

Table 1  
 $\text{Ca}^{2+}$ -induced contraction in rat aorta rings depolarised with 40 mM  $\text{K}^+$  PSS in the presence of nifedipine, BHA and structurally related compounds  
Figures are means  $\pm$  S.E.M. ( $n = 3-16$ ).

Treatment		pEC <sub>50</sub> (M)	Maximum response (mg)
Ethanol	17.4 mM	2.830 $\pm$ 0.080	598.3 $\pm$ 45.7
Nifedipine	5 pM	2.754 $\pm$ 0.017	651.3 $\pm$ 11.8
	50 pM	2.131 $\pm$ 0.411 <sup>a</sup>	149.5 $\pm$ 72.6 <sup>b</sup>
DMSO	14 mM	2.918 $\pm$ 0.053	555.4 $\pm$ 15.8
BHA	50 $\mu\text{M}$	3.021 $\pm$ 0.066	403.4 $\pm$ 24.9 <sup>b</sup>
	200 $\mu\text{M}$	2.453 $\pm$ 0.150 <sup>b</sup>	344.5 $\pm$ 58.5 <sup>b</sup>
BHQ	0.5 $\mu\text{M}$	2.657 $\pm$ 0.053 <sup>a</sup>	310.0 $\pm$ 8.7 <sup>b</sup>
	5 $\mu\text{M}$	2.390 $\pm$ 0.035 <sup>b</sup>	171.3 $\pm$ 3.8 <sup>b</sup>
	50 $\mu\text{M}$	2.671 $\pm$ 0.024 <sup>a</sup>	297.3 $\pm$ 8.1 <sup>b</sup>
NDGA	10 $\mu\text{M}$	3.020 $\pm$ 0.090	361.2 $\pm$ 28.9 <sup>b</sup>
	50 $\mu\text{M}$	2.680 $\pm$ 0.022 <sup>b</sup>	147.1 $\pm$ 3.5 <sup>b</sup>
Propofol	50 $\mu\text{M}$	3.132 $\pm$ 0.108	386.6 $\pm$ 32.9 <sup>b</sup>
	100 $\mu\text{M}$	2.992 $\pm$ 0.032	294.5 $\pm$ 8.8 <sup>b</sup>
DTBHA	50 $\mu\text{M}$	2.941 $\pm$ 0.026	451.7 $\pm$ 11.8 <sup>b</sup>
	100 $\mu\text{M}$	2.477 $\pm$ 0.029 <sup>b</sup>	290.9 $\pm$ 11.2 <sup>b</sup>

<sup>a</sup> $P < 0.05$ , with respect to vehicle alone (Dunnett's test).

<sup>b</sup> $P < 0.01$ , with respect to vehicle alone (Dunnett's test).

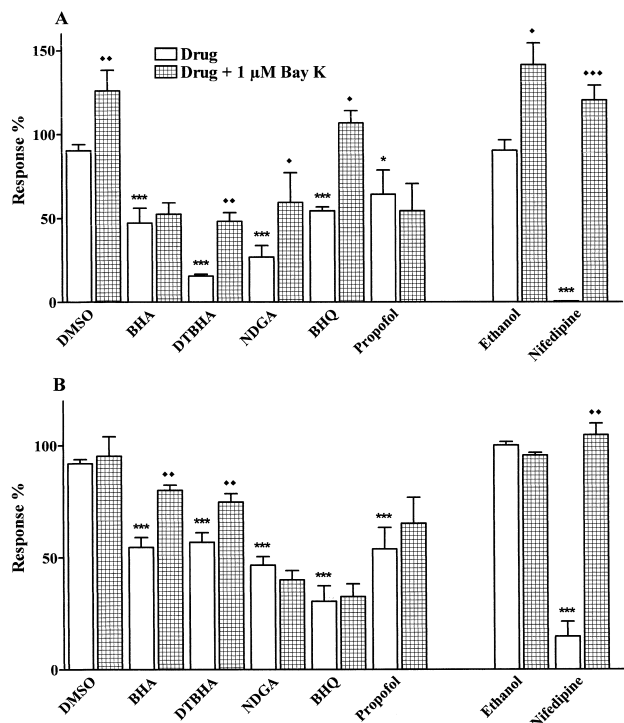


Fig. 2. Inhibition of (A)  $\text{Ca}^{2+}$ - and (B)  $\text{Ba}^{2+}$ -induced contractions in rat aorta rings and its reversal by Bay K 8644. Responses (%) were calculated with respect to control. Columns represent means  $\pm$  S.E.M. ( $n = 3-13$ ). BHA, DTBHA, NDGA, BHQ, propofol (50  $\mu\text{M}$ ) in 14 mM DMSO (final concentration) and 25 nM nifedipine in 17.4 mM ethanol (EtOH) were added 15 min before 1 mM  $\text{Ca}^{2+}$  or 5 mM  $\text{Ba}^{2+}$ ; 1  $\mu\text{M}$  Bay K 8644 was added 5 min before  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ . \* $P < 0.05$ , \*\*\* $P < 0.001$ , with respect to vehicle alone (Dunnett's test for DMSO and Student's  $t$ -test for ethanol); ♦  $P < 0.05$ , ♦♦  $P < 0.01$ , ♦♦♦  $P < 0.001$ , with respect to drug alone (Student's  $t$ -test).

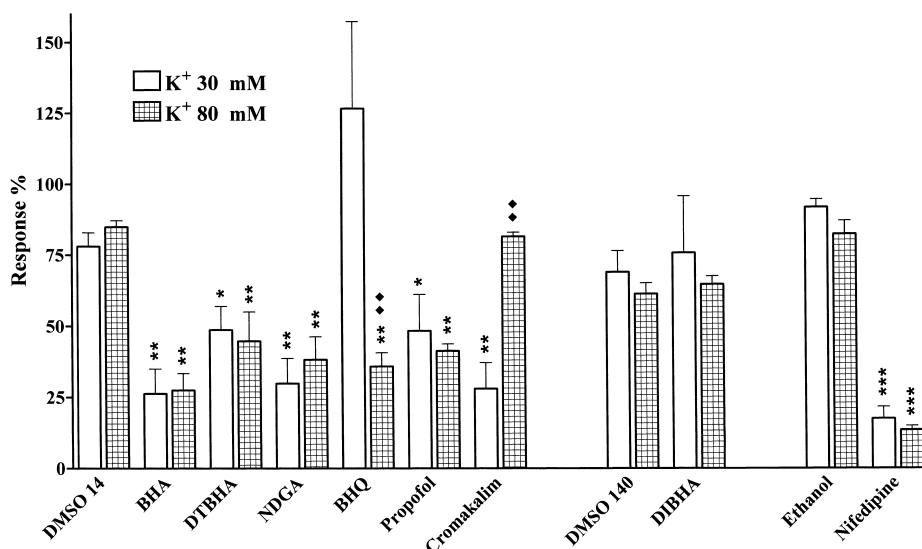


Fig. 3. Effects of various agents on  $\text{Ca}^{2+}$ -dependent contractions at 30 and 80 mM extracellular  $\text{K}^{+}$  concentrations.  $\text{Ca}^{2+}$ -induced contractions were obtained in  $\text{Ca}^{2+}$ -free PSS containing 30 or 80 mM  $\text{K}^{+}$ . Responses (%) were calculated with respect to control. Columns represent means  $\pm$  S.E.M. ( $n = 3-14$ ). BHA, DTBHA, NDGA, BHQ, propofol (50  $\mu\text{M}$ ) and cromakalim (0.5  $\mu\text{M}$ ) in 14 mM DMSO (final concentration), DIBHA (50  $\mu\text{M}$ ) in 140 mM DMSO, and 25 nM nifedipine in 17.4 mM ethanol (EtOH) were added 15 min before 1 mM  $\text{Ca}^{2+}$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , with respect to vehicle alone (Dunnett's test for DMSO 14 and Student's  $t$ -test for DMSO 140 and EtOH); ♦♦♦  $P < 0.001$ , with respect to 30 mM  $\text{K}^{+}$  (Student's  $t$ -test).

Instat version 3.00 (GraphPad Software, CA, USA).  $P$  values  $< 0.05$  were considered significant. The pharmacological response to each substance, described in terms of  $\text{pEC}_{50}$  (the negative logarithm, base 10, of  $\text{EC}_{50}$ ), was calculated using GraphPad Prism version 3.01 (GraphPad Software).

### 3. Results

#### 3.1. Endothelium-dependent relaxation

In endothelium-intact aorta rings, BHA, DTBHA, NDGA, DIBHA and propofol, added at the plateau the  $\text{EC}_{90}$  ( $\text{pEC}_{90} = 5.782 \pm 0.085$ ,  $n = 24$ ) phenylephrine-evoked contraction, neither showed spasmolytic activity nor affected the concentration–relaxation curve for acetylcholine (data not shown).

#### 3.2. Concentration–response curve for $\text{Ca}^{2+}$

Table 1 shows the effects of BHA, DTBHA, BHQ, NDGA, propofol and nifedipine on the contraction induced by cumulative addition of  $\text{Ca}^{2+}$  (0.05–10 mM) to aorta rings depolarised with 40 mM  $\text{K}^{+}$  PSS. All compounds but propofol significantly reduced the  $\text{pEC}_{50}$  for  $\text{Ca}^{2+}$ , at least at the highest concentration tested; a significant reduction in maximum response was also observed. Notably, BHQ showed bell-shaped antagonism with maximum inhibition at 5  $\mu\text{M}$ . DIBHA had no effect on this model (data not shown). The inhibition exerted by 200  $\mu\text{M}$  BHA was

reduced by a 10-fold increase in extracellular  $\text{Ca}^{2+}$  concentration (from 73.0% of control, at 1 mM  $\text{Ca}^{2+}$ , to 44.9% at 10 mM  $\text{Ca}^{2+}$ ).

### 3.3. $\text{Ca}^{2+}$ - and $\text{Ba}^{2+}$ -induced contraction

Fig. 2A shows the effects of the test compounds on the contraction elicited by 1 mM  $\text{Ca}^{2+}$  in 40 mM  $\text{K}^+$  PSS in the presence or absence of 1  $\mu\text{M}$  Bay K 8644. It can be seen that the  $\text{Ca}^{2+}$ -induced contraction was completely inhibited by 25 nM nifedipine and that the presence of Bay K 8644 restored the response to control values.

DTBHA, NDGA, BHA, BHQ and propofol (50  $\mu\text{M}$ ) significantly inhibited the  $\text{Ca}^{2+}$ -evoked contraction by 84.4%, 73.0%, 52.8%, 45.6% and 35.7%, respectively. Bay K 8644 reversed their antispasmodic effects to differ-

ent degrees: complete restoration in the case of BHQ, marked but still significantly lower than control values in the case of DTBHA and NDGA, no effect in the case of BHA and propofol.

Fig. 2B shows the effects of the test compounds on the contractile response to 5 mM  $\text{Ba}^{2+}$ . The  $\text{Ba}^{2+}$ -induced contraction was sharply inhibited by 25 nM nifedipine (85.2%) and its effect was totally reversed by 1  $\mu\text{M}$  Bay K 8644. BHA and DTBHA (50  $\mu\text{M}$ ) revealed antispasmodic activity (45.3% and 43.1%, respectively) which was partly reversed by Bay K 8644 (19.9% and 25.2%, respectively). In contrast, Bay K 8644 did not modify the inhibition exerted by BHQ, NDGA and propofol (69.5%, 53.3% and 46.1%, respectively).

### 3.4. Assessment of $\text{K}^+$ channel opening activity

As shown in Fig. 3, a change in external  $\text{K}^+$  concentration did not modify the BHA, DTBHA, NDGA or nifedipine inhibition of the  $\text{Ca}^{2+}$ -induced contraction. In contrast, the relaxant effect of cromakalim appeared only at 30 mM  $\text{K}^+$  and that of BHQ only at 80 mM  $\text{K}^+$ .

When  $^{86}\text{Rb}^+$  efflux was measured in the presence of BHA or cromakalim, only the latter significantly stimulated  $^{86}\text{Rb}^+$  efflux from the ring preparations, with a maximum of 70% over initial values within 4 min. BHA did not modify the basal  $^{86}\text{Rb}^+$  efflux rate.

### 3.5. Contraction induced by mobilization of $\text{Ca}^{2+}$ from intracellular stores

The amount of  $\text{Ca}^{2+}$  stored intracellularly was estimated from the amplitude of the phenylephrine-induced contraction in 80 mM  $\text{K}^+$  PSS and in the presence of 1  $\mu\text{M}$  nifedipine. Only 10  $\mu\text{M}$  papaverine significantly affected the phenylephrine-induced contraction (Fig. 4A). However, when  $\text{Ni}^{2+}$  was present in the bath solution, BHQ also significantly inhibited the tissue response to phenylephrine (Fig. 4B).

## 4. Discussion

Depolarisation of smooth muscle increases cell membrane permeability to  $\text{Ca}^{2+}$  by opening membrane voltage-dependent  $\text{Ca}^{2+}$  channels:  $\text{Ca}^{2+}$  enters the cell, causing contraction. Smooth muscle relaxation can be achieved by blockade of  $\text{Ca}^{2+}$  entry or enhancement of  $\text{K}^+$  efflux through plasmalemmal  $\text{K}^+$  channels. The latter results in hyperpolarization of the membrane: voltage-dependent  $\text{Ca}^{2+}$  channels close,  $\text{Ca}^{2+}$  does not enter the cell and relaxation occurs. According to this clearly established scheme, the possible mechanism(s) underlying the antispasmodic activity of BHA were investigated in rat aorta rings.

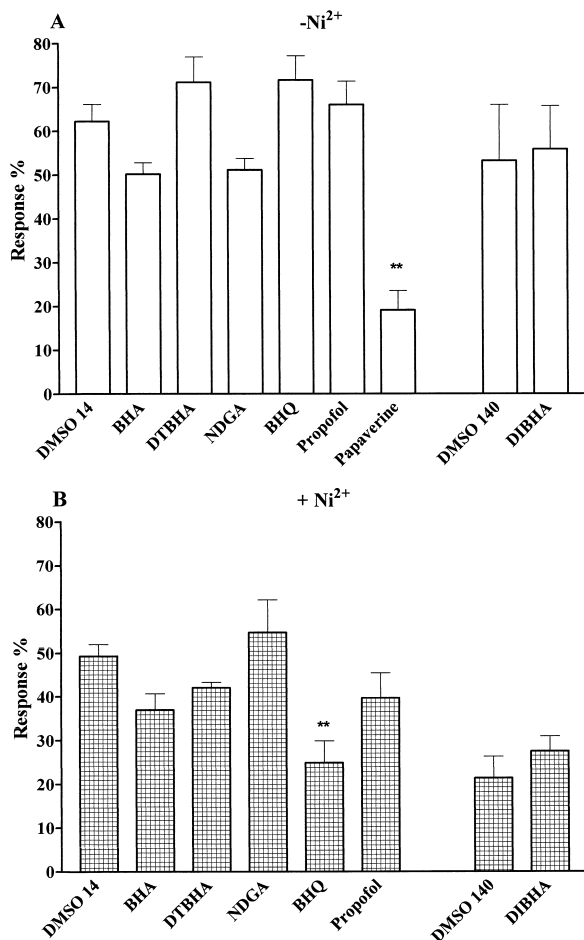


Fig. 4. Effects of various agents on  $\text{Ca}^{2+}$  mobilisation from intracellular stores. The rings were stimulated with 1  $\mu\text{M}$  phenylephrine in 80 mM  $\text{K}^+$  PSS containing 1  $\mu\text{M}$  nifedipine alone (A) or nifedipine plus 1 mM  $\text{Ni}^{2+}$  (B). This response was assumed to indicate the contribution of intracellular  $\text{Ca}^{2+}$  sources to the contractile response. Columns represent means  $\pm$  S.E.M. ( $n = 3-15$ ). BHA, DTBHA, NDGA, BHQ, propofol (50  $\mu\text{M}$ ) and papaverine (10  $\mu\text{M}$ ) in 14 mM DMSO (final concentration), and DIBHA (50  $\mu\text{M}$ ) in 140 mM DMSO were added 15 min before phenylephrine. \*\* $P < 0.01$ , with respect to vehicle alone (Dunnett's test for DMSO 14 and Student's  $t$ -test for DMSO 140).

BHA inhibited the  $\text{Ca}^{2+}$ -induced contraction obtained under different depolarising conditions (i.e. 30, 40 as well as 80 mM  $\text{K}^+$ ), similarly to the  $\text{Ca}^{2+}$ -channel blocking drug nifedipine. Marked antispasmodic activity of BHA was also demonstrated in the case of  $\text{Ba}^{2+}$ -induced contraction, as observed previously in intestinal smooth muscle (Sgaragli et al., 1993b). The antispasmodic activity exerted by BHA was found to be affected to different extents by the dihydropyridine agonist of L-type  $\text{Ca}^{2+}$  channels, Bay K 8644. In fact, complete reversal of the effect of BHA by Bay K 8644 was only achieved for  $\text{Ba}^{2+}$ -induced contraction and not for  $\text{Ca}^{2+}$ -induced contraction. In the presence of Bay K 8644, the antispasmodic effect of nifedipine disappeared in both models. In the presence of BHA, the concentration–response curves for  $\text{Ca}^{2+}$  shifted to the right and a marked reduction in maximum response was observed. Moreover, the inhibition was reduced by a 10-fold increase in extracellular  $\text{Ca}^{2+}$  concentrations, suggesting that BHA competes with  $\text{Ca}^{2+}$  for entry into the open channel.

When we checked the antispasmodic activity of the test compounds at different depolarising levels, it emerged that an increase in extracellular  $\text{K}^+$  concentration caused a decrease in the antispasmodic activity of cromakalim, a  $\text{K}^+$  channel opener (Norman et al., 1994). This is reasonable because during exposure to 80 mM  $\text{K}^+$ , the  $\text{K}^+$  gradient across the membrane drops drastically and the effect of  $\text{K}^+$  channel opening disappears (Gurney, 1994). In contrast, BHA revealed marked antispasmodic activity both at high (80 mM  $\text{K}^+$ ) and low (30 mM  $\text{K}^+$ ) depolarising levels. Furthermore, by measuring  $^{86}\text{Rb}^+$  efflux, we showed that cromakalim, but not BHA, stimulated  $\text{K}^+$  efflux. Taken together, these experimental results indicate that BHA is more likely to have  $\text{Ca}^{2+}$  blocking activity than  $\text{K}^+$  channel opening activity. This is also supported by data from this laboratory showing partial inhibition of  $\text{Ca}^{2+}$  currents in rat tail artery smooth muscle cells by BHA (Petkov et al., 1999). Moreover, the present experiments designed to establish the contribution of intracellular  $\text{Ca}^{2+}$  stores demonstrated that BHA did not modify agonist-induced  $\text{Ca}^{2+}$  release from intracellular stores.

NO can react with methyl- or *t*-butyl-substituted phenols to give a phenoxyl radical which subsequently couples reversibly with excess NO (Janzen et al., 1993), thus functioning as a NO carrier in biological systems. However, BHA did not modify the acetylcholine-induced relaxation of precontracted rings, nor did it affect phenylephrine-induced tone. This suggests that BHA neither acts as a NO carrier/amplifier nor releases NO from the endothelium.

Two additional targets should be taken into account when discussing smooth muscle relaxation: mitochondria and cyclic nucleotides. The former were recently demonstrated to play a role in removing  $\text{Ca}^{2+}$  from the cytosol after stimulation (Drummond and Fay, 1996). Previous studies (Fusi et al., 1991, 1992) showed that BHA affected

liver mitochondrial function at a concentration of 100  $\mu\text{M}$ . BHA myorelaxant activity could therefore be partly due to its effects on energy metabolism. However, additional experiments are necessary to verify this hypothesis. Cyclic nucleotides control several cell functions as well as vascular tone (Lincoln and Cornwell, 1991). Drugs that increase cyclic nucleotide levels cause relaxation of agonist-induced contraction in smooth muscle, as observed for BHA in the gastric fundus (Fusi et al., 1998b). In rat aorta rings, papaverine, an inhibitor of cAMP phosphodiesterase, inhibited phenylephrine-induced contraction (see also Karaki, 1987), whereas BHA had no effect, thus excluding any involvement of cyclic nucleotides in the mechanism of action.

Modification of the molecular structure of BHA by the introduction of an additional *t*-butyl group in the *o*- or *m*-position of the hydroxyl group, and further demethylation of the methoxy group, as seen for DTBHA and BHQ, respectively, gave rise to a more selective blocker of L-type  $\text{Ca}^{2+}$  channels (Fusi et al., 1998a; Petkov et al., 1999). In fact, the BHQ inhibition of the  $\text{Ca}^{2+}$ -induced contraction, at a high  $\text{K}^+$  concentration, was completely reversed by Bay K 8644 and augmented by increasing membrane depolarisation (Gurney, 1994). The myorelaxant effect of BHQ, however, is rather complex and involves endothelial functions (Fusi et al., 1998a, 1999) which might contribute to the final effect observed here. Simplification of the BHA structure to obtain propofol did not greatly change the activity of the parent compound, suggesting that the hydroxyl group structurally hindered by a bulky lipophilic moiety is a necessary requirement for antispasmodic activity, as stated before (Korn and Horn, 1990; Sgaragli et al., 1993b). With the exception of BHQ, none of the tested compounds affected endothelial function or agonist-induced release of  $\text{Ca}^{2+}$  from intracellular stores, again suggesting that minor structural alterations have marked effects on the pharmacological activity of this class of molecules. In this context, it is worth considering the case of DIBHA, since dimerization of BHA caused a total loss of its antispasmodic activity. DIBHA, a stable product of intestinal peroxidase metabolism of BHA (Valoti et al., 1989), has no inhibitory effect on mitochondrial oxidative phosphorylation (Fusi et al., 1991), is not toxic to gut musculature when administered i.p. (Sgaragli et al., 1993a) and does not inhibit  $\text{Ba}^{2+}$ -induced contraction in ileal longitudinal smooth muscle, though it retains the antioxidant activity of the parent compound (Sgaragli et al., 1993b). This change in properties, following dimerization, is unlikely to be due to decreased partitioning of DIBHA into membranes, because there is little change in hydrophobicity on dimerization. Furthermore, molecular size alone cannot account for the loss of antispasmodic activity, as can be seen with NDGA. Though similar in size, NDGA is reported to inhibit  $\text{Ca}^{2+}$  channel currents in GH3 and AtT-20 pituitary cells, at concentrations similar to those used here (Korn and Horn, 1990).

In conclusion, BHA exerts non-specific inhibition on  $\text{Ca}^{2+}$  influx in vascular smooth muscle by a mechanism distinct from that of other known  $\text{Ca}^{2+}$  channel blockers. Although BHA apparently competes with  $\text{Ca}^{2+}$ , other experiments are necessary to determine its precise mechanism of action.

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