ELSEVIER

European Journal of Pharmacology 360 (1998) 43–50

ejp

Myorelaxant activity of 2-*t*-butyl-4-methoxyphenol (BHA) in guinea pig gastric fundus

Fabio Fusi, Massimo Valoti, Georgi V. Petkov [1], Kiril K. Boev [1], Gian P. Sgaragli *

Istituto di Scienze Farmacologiche, Università di Siena, via E.S. Piccolomini 170, 53100 Siena, Italy

Received 13 July 1998; revised 31 August 1998; accepted 4 September 1998

Abstract

This study investigates the mechanism whereby the antioxidant 2-*t*-butyl-4-methoxyphenol (BHA) relaxes guinea pig gastric fundus smooth muscle. In circular smooth muscle strips, 10 μM cyclopiazonic acid, a specific inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase, induced a prolonged rise in tension which depended on the presence of extracellular Ca^{2+}. BHA ($pIC_{50} = 5.83$), sodium nitroprusside (6.85), isoproterenol (7.69) and nifedipine (8.02), but not 2,6-di-*t*-butyl-4-methoxyphenol (DTBHA) (up to 30 μM), relaxed muscle strips contracted with cyclopiazonic acid. Methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) (1 μM) antagonised the nifedipine- but not the BHA-induced relaxation. Nifedipine and isoproterenol (10 μM) caused a decrease in spontaneous tone, but did not counteract the subsequent rise in tension elicited by 10 μM cyclopiazonic acid. Conversely, 100 μM BHA and 100 μM sodium nitroprusside not only significantly reduced spontaneous tone but also markedly impaired the response of the muscles to cyclopiazonic acid. DTBHA failed to show either effect. When added to preparations completely relaxed by 100 μM BHA, 10 mM tetraethylammonium still elicited nifedipine-sensitive tonic and phasic contractions in the presence or absence of 10 μM cyclopiazonic acid. BHA and DTBHA inhibited, in a concentration-dependent manner, the Ca^{2+}-promoted contraction of strips depolarised by 10 mM tetraethylammonium. The BHA antagonism showed a non-competitive profile while that of DTBHA was competitive. In muscle strips at rest, 10 μM BHA caused a significant increase in tissue cAMP concentration, leaving cGMP unmodified. To conclude, the myorelaxant action of BHA on gastric fundus smooth muscle appears to be mediated partly by an increase in cAMP levels and partly by inhibition of Ca^{2+} influx from the extracellular space.

Keywords: BHA (2-*t*-butyl-4-methoxyphenol); DTBHA (2,6-di-*t*-butyl-4-methoxyphenol); Cyclopiazonic acid; Sarcoplasmic reticulum Ca^{2+}-ATPase; cAMP

1. Introduction

Research on cell protection after ischemia-reperfusion injury has recently been focused on a dual pharmacological strategy which intervenes in the various phases of the pathological event, i.e., before, during and after ischemia: inactivating oxyradicals with antioxidants and limiting the availability of free intracellular Ca^{2+} by reducing its influx from the extracellular space.

From a pharmacological viewpoint, therefore, molecules which combine both scavenging and Ca^{2+} channel antagonist properties 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^{2+} overload. In a recent attempt to identify such dual-purpose molecules, we demonstrated the dual action of fifteen phenol derivatives: they had an antispasmogenic action on longitudinal ileum musculature and antioxidant activity in microsomal and linoleate systems (Sgaragli et al., 1993). The same study showed that compounds with a phenol moiety sterically hindered by a lipophilic group, such as the antioxidants 2-*t*-butyl-4-methoxyphenol (BHA) and 2,6-di-*t*-butyl-4-methoxyphenol (DTBHA), exert their spasmolytic action via inhibition of Ca^{2+} influx into cells through L-type Ca^{2+} channels.

BHA has been shown to inhibit agonist evoked increases in cytosolic Ca^{2+} concentrations (Alexandre et al., 1986). Zoccarato et al. (1987) showed that, under depolarising conditions, BHA strongly inhibits Ca^{2+} uptake by guinea pig cerebral cortex synaptosomes and the subsequent γ-aminobutyric acid (GABA) and glutamate release. Furthermore, hydroxyl or peroxyl radical scavengers have

* Corresponding author. Tel.: +39-577-221-255/48724; Fax: +39-577-281-928; E-mail: sgaragli@unisi.it

[1] Present address: Institute of Biophysics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria.

0014-2999/98/$ - see front matter
PII: S0014-2999(98)00660-8

recently proved useful in the prevention of experimental ischemia-reperfusion damage. Such compounds include the novel butylated hydroxytoluene derivatives, 5-[[3, 5-bis[1,1-dimethylethyl]-4-hydroxyphenyl]-methylene]--3-methyl-4-thiazolidinone (LY256548) (Ruterbories and Lindstrom, 1990), *p*-(pyrrolidinylmethylene)butylated hydroxytoluene (E-5110) (Shirota et al., 1987) and 2-(allyl-1-piperazinyl)-4-*n*-amyl-oxyquinazoline fumarate (KB-5666) (Hara and Kogure, 1990).

Although their antioxidant efficacy has long been known, the potential protection afforded by the interaction of these hindered phenols with Ca^{2+} homeostasis is still a very new field of investigation. We therefore examined the pharmacodynamics of the two most effective representatives of this bifunctional class of compounds, BHA and DTBHA, by analysing their spasmolytic and antispasmogenic activities in circular smooth muscle strips isolated from the guinea pig gastric fundus. These muscle fibres have an efficient Ca^{2+} uptake system which functions through sequestering sites presumably situated just beneath the muscle membrane (Kuriyama et al., 1976). The gastric fundus responds to external stimuli with spike-free tonic contractions. Tetraethylammonium, a non-specific K^+ channel blocker, and cyclopiazonic acid, a specific inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase (Seidler et al., 1989), were used as pharmacological tools in the present study. In vascular smooth muscle, the peripherally located sarcoplasmic reticulum (Bond et al., 1984) accumulates Ca^{2+} entering the plasmalemma and functions as a 'buffer barrier' against its further penetration into the deeper myoplasm (van Breemen et al., 1995) where it promotes contraction. The model of a 'superficial buffer barrier' has also been confirmed for the gastric fundus smooth muscle preparation used here (Petkov and Boev, 1996). Disruption of this 'superficial buffer barrier' by an agent such as cyclopiazonic acid leads to an increase in $[Ca^{2+}]_i$. The ensuing muscle contraction will therefore be the result of the interplay between the sarcoplasmic reticulum Ca^{2+} leak and the opening of plasmalemmal Ca^{2+} channels (Putney, 1990).

The results reported here indicate that the antispasmogenic effect of BHA on guinea pig gastric fundus can be attributed to the increase in intracellular cAMP concentration ($[cAMP]_i$) as well as to the block of plasmalemmal Ca^{2+} channels. These effects appear to be fairly specific in view of the different pharmacological profile of a strict structural analogue, DTBHA, which induces muscle relaxation by inhibiting Ca^{2+} influx through the cell membrane.

2. Materials and methods

2.1. Tissue preparation and contractility studies

Adult male guinea pigs (350–450 g), fasted overnight, were anaesthetised with a mixture of Ketavet® and Rompum®, decapitated and exsanguinated. The stomach was removed, opened along the longitudinal axis of the greater curvature and washed in cold Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS) containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25 and glucose 11.5. The stomach was then pinned flat in a dissecting dish filled with Ca^{2+}-free PSS, and smooth muscle strips (1–2 mm in width; 1.5–2 cm in length) were dissected from the circular layer of the anterior fundus wall (upper part), avoiding mucosa contamination, and transferred into 20-ml organ bath chambers filled with Ca^{2+}-free PSS at 37°C, gassed with 5% CO_2–95% O_2, pH 7.4. Strips were connected to isometric transducers (Basile, Varese, Italy) and stretched to a tension of 10 mN. After equilibration for 15 min, Ca^{2+}-free PSS was replaced with normal PSS containing 2.5 mM Ca^{2+} and the strips were allowed to develop stable spontaneous tone over a 30-min period.

At the end of each experimental session, 100 μM sodium nitroprusside was added to suppress any residual contraction. Under sodium nitroprusside the strips reached a resting tone of zero tension, equivalent to that achieved when Ca^{2+} was omitted from the bath.

Dimethyl sulfoxide and ethanol, at the maximum concentration which bathed tissues (0.18% and 0.11%, v/v, respectively), affected neither spontaneous tone nor the response to the drugs.

2.2. Experiments in the presence of cyclopiazonic acid

In a first series of experiments, 10 μM cyclopiazonic acid was added to the bath and maximum, stable contractions developed over a 20–60-min period. Drugs were then added and cumulative concentration-response curves were plotted. The compounds tested as spasmolytic agents (BHA, DTBHA, nifedipine, isoproterenol and sodium nitroprusside), with or without the addition of tetrodotoxin and/or atropine, were left in contact with the strips, at each concentration used, for long enough to allow full development of the effect.

In a second series of experiments cyclopiazonic acid-elicited tension was studied in strips preincubated for 15 min with various antispasmogens.

In some experiments, the dihydropyridine receptor agonist methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) was added together with cyclopiazonic acid to determine whether the ability of BHA or nifedipine to reverse the cyclopiazonic acid-promoted contraction depended on impairment of L-type Ca^{2+} channels. The strip was subjected to cumulative addition of increasing concentrations of BHA (or nifedipine), after cyclopiazonic acid, in order to test relaxation effects. After several washes with normal PSS, spontaneous tone reverted to control values. The same preparation was then exposed to 1 μM Bay K 8644 and 10 μM cyclopiazonic acid. When the plateau contrac-

tion was reached after 30–60 min (a time sufficient to obtain full Bay K 8644 activation of L-type Ca^{2+} channels), BHA or nifedipine was administered cumulatively.

In a few experiments, 30 μM ryanodine was used to empty intracellular Ca^{2+} stores; it was added 45 min before cyclopiazonic acid due to its slow onset of action (Bourreau et al., 1991).

2.3. Experiments in the presence of tetraethylammonium and Ba^{2+}

These experiments were performed in the presence of 0.1 μM tetrodotoxin to avoid any neurotransmitter release caused by K^+ channel blockers (Boev et al., 1985).

In a first series of experiments, the effects of tetraethylammonium and Ba^{2+} were tested on strips preincubated with BHA, with and without cyclopiazonic acid. Nifedipine was added at the end of all experiments.

In a second series, 10 mM tetraethylammonium was added to the bath and a maximum, stable contraction developed within 20 min. BHA was then added to obtain a cumulative concentration-response relaxation.

In a third series, after three similar consecutive responses were obtained to 10 μM acetylcholine, PSS was replaced with Ca^{2+}-free PSS containing 10 mM tetraethylammonium. The spasmogenic response to Ca^{2+} (0.03–3 mM) was studied by recording cumulative concentration-effect curves. An initial concentration-effect curve was obtained for Ca^{2+} followed by two others. In test tissues, BHA or DTBHA was present for 15 min before and throughout recording of the second and third concentration-effect curves; their concentration was increased 15 min before the third curve. Control tissues were treated similarly but without exposure to BHA and DTBHA. In both control and test conditions, cumulative concentration-effect curves for Ca^{2+} were drawn on experimental data normalised against the response to 3 mM Ca^{2+} (taken as 100%) of the initial curve.

2.4. Measurement of cyclic nucleotides

After the equilibration period (see above), strips were incubated for 15 min with dimethyl sulfoxide (control), 10 μM BHA or 10 μM sodium nitroprusside. The strips were then homogenised in 1 ml PSS containing 50 μM 3′-isobutyl-1-methylxanthine and 10 μM indomethacin. Then 500 μl of ice-cold trichloroacetic acid (10% w/v) was added and the samples were stored under liquid nitrogen until measurement. The cyclic nucleotides were extracted from trichloroacetic acid with 0.5 M tri-*n*-octylamine (Harper and Brooker, 1975) and the concentrations of cGMP and cAMP in the aqueous phase were measured with a radioimmunoassay kit using ^{125}I-labelled cGMP and ^{3}H-labelled cAMP (Steiner et al., 1972; Salvemini et al., 1991). The recovery of cGMP and cAMP was about 99%, so the results were not corrected for loss (Salvemini et al., 1991). All the determinations were performed in quadruplicate. The results are expressed as fmol of cGMP and pmol of cAMP per mg of protein. The protein concentrations were determined by the Lowry method, using bovine serum albumin as standard (Lowry et al., 1951).

2.5. Drugs: commercial sources and solutions

BHA from Fluka Chemie (Buchs, Switzerland) was recrystallised once from petroleum ether prior to use. DTBHA from Aldrich-Chemie (Steinheim, Germany) was recrystallised once from ethanol prior to use. Nifedipine, Bay K 8644, cyclopiazonic acid, tetrodotoxin, tetraethylammonium, EGTA, tri-*n*-octylamine, 3′-isobutyl-1-methylxanthine, indomethacin, isoproterenol and 1,1,2-trichlorotrifluoroethane were purchased from Sigma (Milan, Italy). Calbiochem (La Jolla, CA, USA) supplied ryanodine. Atropine was purchased from Merck (Darmstadt, Germany). Sodium nitroprusside was obtained from Riedel-De Haën (Seelze-Hannover, Germany). Kits for the radioimmunoassay of cGMP and cAMP were purchased from Amersham (Amersham, UK). All other compounds were of analytical grade and were used without further purification.

Stock solutions of cyclopiazonic acid, BHA, DTBHA and ryanodine in 100% dimethyl sulfoxide, and nifedipine

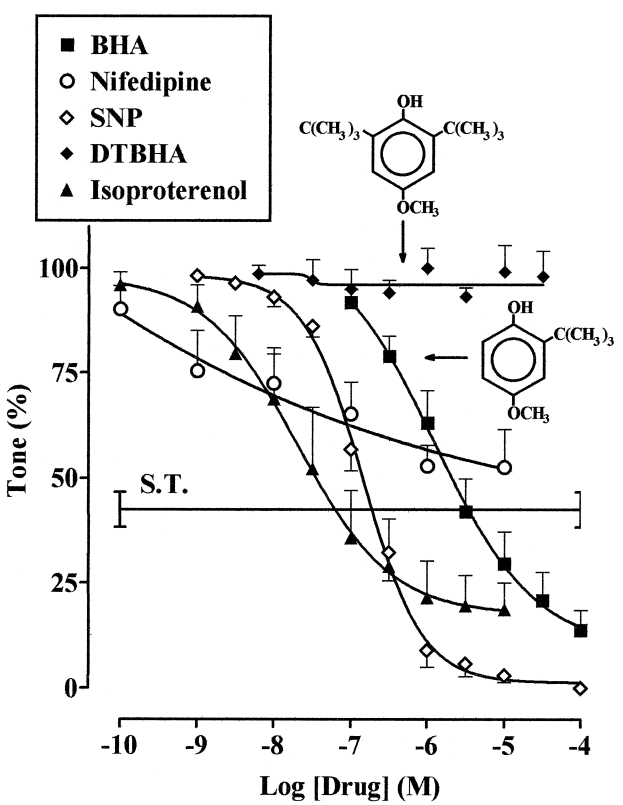

Fig. 1. Relaxant responses induced by BHA, isoproterenol, sodium nitroprusside (SNP) and nifedipine, but not by DTBHA, in guinea pig gastric fundus strips contracted with 10 μM cyclopiazonic acid. Data points are means and vertical lines represent S.E.M. ($n = 3–9$) of the response to 10 μM cyclopiazonic acid taken as 100%. S.T. = spontaneous tone.

and Bay K 8644 in absolute ethanol were stored at −20°C and shielded from light with aluminium foil.

Water for solutions was first distilled and then passed through a NANOpure II deionisation system (Barnstead-Sybron, Boston, MA, USA), to obtain Type I Reagent Grade water (resistivity 18 MΩ). Concentrations refer to the final concentrations in the bath chambers.

2.6. Statistical analysis

Data are presented as means ± S.E.M.; n is the number of animals. Statistical analysis was performed by using Student's t-test for paired samples or a one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. P values < 0.05 were considered significant. The pharmacological response to each substance was described in terms of either pIC_{50} or pEC_{50}.

3. Results

3.1. Effect of cyclopiazonic acid

In normal PSS, cyclopiazonic acid induced a prolonged concentration-dependent rise in tension with a pEC_{50} of 5.39 ± 0.04 ($n = 10$). The tension developed with 10 μM cyclopiazonic acid (241.6 ± 22.5% of spontaneous tone, $n = 10$) and spontaneous tone were abolished after addition of 3 mM EGTA (1.1 ± 1.1%, $n = 3$; $P < 0.01$). Strips incubated with 10 μM cyclopiazonic acid in Ca^{2+}-free PSS containing 100 μM EGTA not only failed to contract but lost spontaneous tone (5.8 ± 3.3%, $n = 3$; $P < 0.01$).

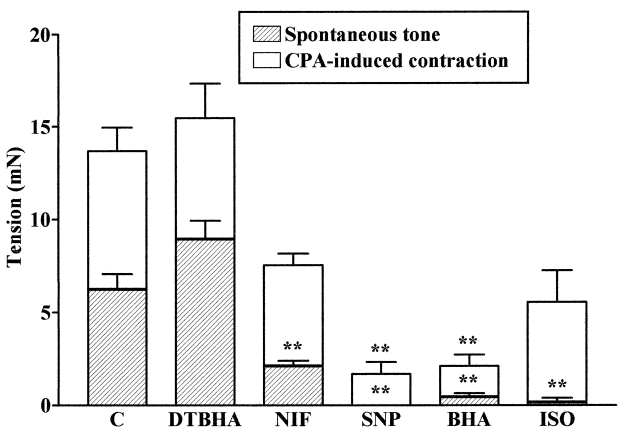

Fig. 2. Effect of cyclopiazonic acid on strips incubated with various agents. DTBHA (30 μM), isoproterenol (ISO) and nifedipine (NIF) (10 μM), sodium nitroprusside (SNP) and BHA (100 μM) were added at IC_{100} concentrations (see Fig. 1) 15 min before the addition of 10 μM cyclopiazonic acid (CPA). Cross-hatched columns show effects on spontaneous tone and open columns effects on the cyclopiazonic acid-promoted contraction. Columns are means and vertical lines represent S.E.M. ($n = 6$–10). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, ** $P < 0.01$ compared to control (C) ($n = 21$).

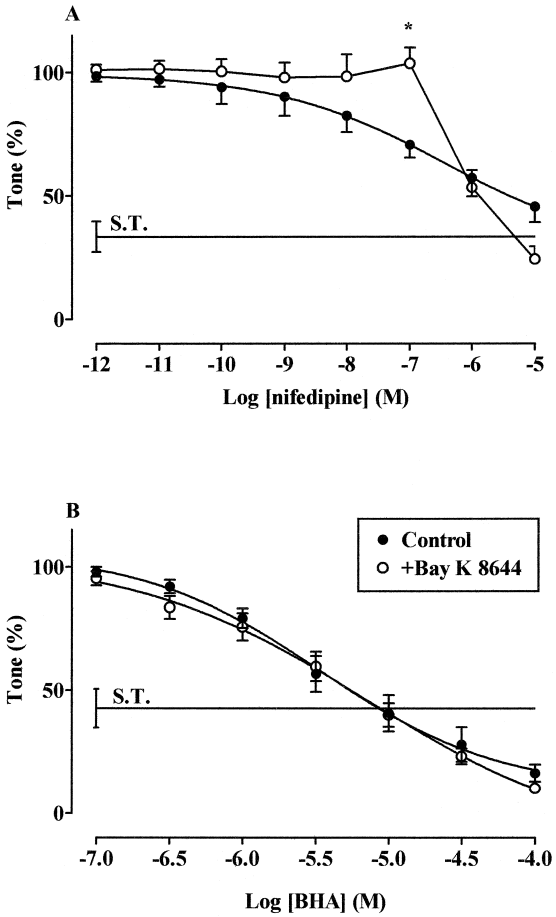

Fig. 3. Relaxant responses induced by nifedipine (A) and BHA (B) in strips contracted with 10 μM cyclopiazonic acid in the absence (Control) or in presence of 1 μM Bay K 8644. Data points are means and vertical lines represent S.E.M. ($n = 6$) of the response to 10 μM cyclopiazonic acid taken as 100%. S.T. = spontaneous tone. Statistical analysis was performed using Student's t-test for paired samples, * $P < 0.05$.

When Ca^{2+} was introduced in concentrations up to 2.5 mM a contraction, similar to that obtained in normal PSS, occurred within 3 min (data not shown). Cyclopiazonic acid-elicited tension in normal PSS was enhanced by 30 μM ryanodine (560.6 ± 56.2%, $n = 3$; $P < 0.01$), but unaffected by 10 μM nifedipine (184.4 ± 24.8%, $n = 3$; $P > 0.05$) and by 10 mM tetraethylammonium (242.8 ± 28.8%, $n = 4$; $P > 0.05$). Similar changes occurred when strips were incubated with 0.1 μM tetrodotoxin and 0.1 μM atropine, either alone or in combination (data not shown).

3.2. Effect of various compounds on cyclopiazonic acid-induced contraction

BHA (pIC_{50} value of 5.83 ± 0.11, $n = 8$), nifedipine (8.02 ± 0.44, $n = 9$), isoproterenol (7.69 ± 0.25, $n = 3$) and sodium nitroprusside (6.85 ± 0.04, $n = 3$) promoted concentration-dependent spasmolysis in strips contracted with 10 μM cyclopiazonic acid. BHA, isoproterenol and

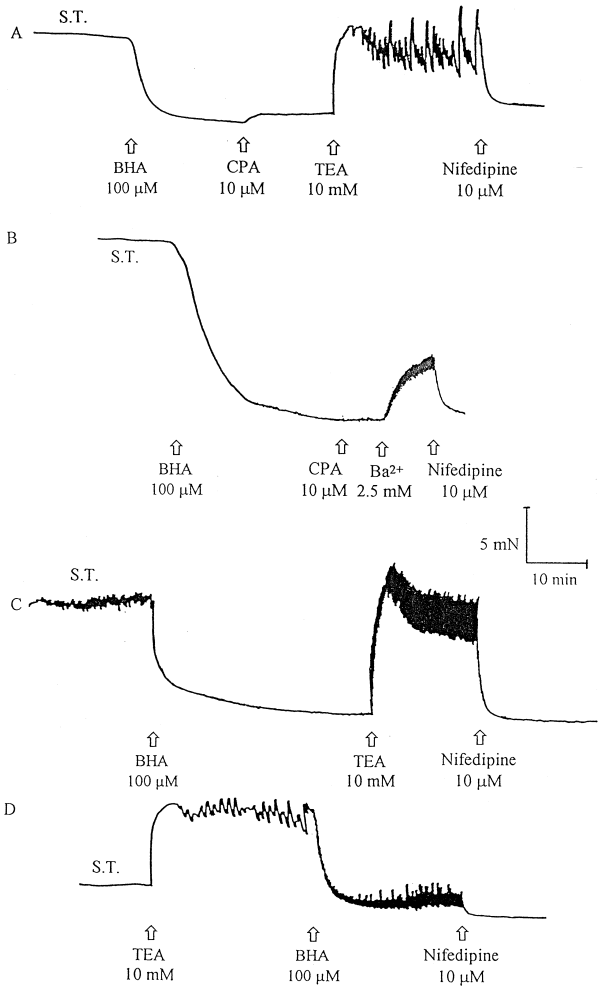

Fig. 4. Effects of BHA, cyclopiazonic acid (CPA), tetraethylammonium (TEA), nifedipine and Ba^{2+} (addition indicated by arrow) on the spontaneous tone of strips. Tracings are representative of experiments repeated at least four times with similar results. At the end of each experimental session, nifedipine was added to suppress any residual contraction. S.T. = spontaneous tone.

sodium nitroprusside (Fig. 1) reversed cyclopiazonic acid-induced tension to far below spontaneous tone, whereas the relaxant effect of nifedipine only brought tension close to the spontaneous tone. In contrast, DTBHA, even at a concentration of 30 μM failed to affect the cyclopiazonic acid-promoted contraction. The concentration-relaxation curve for BHA was unaffected by the addition of 0.1 μM atropine and/or 0.1 μM tetrodotoxin, and by 30 μM ryanodine (data not shown).

It was possible to separate the effect on spontaneous tone from that on cyclopiazonic acid-promoted contraction when the sequence of the additions was changed, i.e., the different compounds were added in IC_{100} concentrations before 10 μM cyclopiazonic acid. As shown in Fig. 2, BHA and sodium nitroprusside reduced spontaneous tone and the cyclopiazonic acid-promoted contraction. In contrast, nifedipine and isoproterenol only affected spontaneous tone, albeit significantly, leaving the cyclopiazonic acid component of the contraction unmodified. DTBHA (30 μM) had no effect on either component.

3.3. Effects of Bay K 8644 on spasmolysis produced by BHA and nifedipine in cyclopiazonic acid-contracted muscle

As shown in Fig. 3, BHA-induced spasmolysis was unaffected by 1 μM Bay K 8644 (Fig. 3B). The Ca^{2+} channel agonist, in fact, only antagonised nifedipine-induced spasmolysis (Fig. 3A). This antagonism was only achieved when the nifedipine to Bay K 8644 concentration ratio was ≤ 1.

3.4. Effects of nifedipine and BHA on muscle contraction elicited by tetraethylammonium and Ba^{2+}

In a first series of experiments, 100 μM BHA markedly reduced the spontaneous tone of the preparation (Fig. 4A,

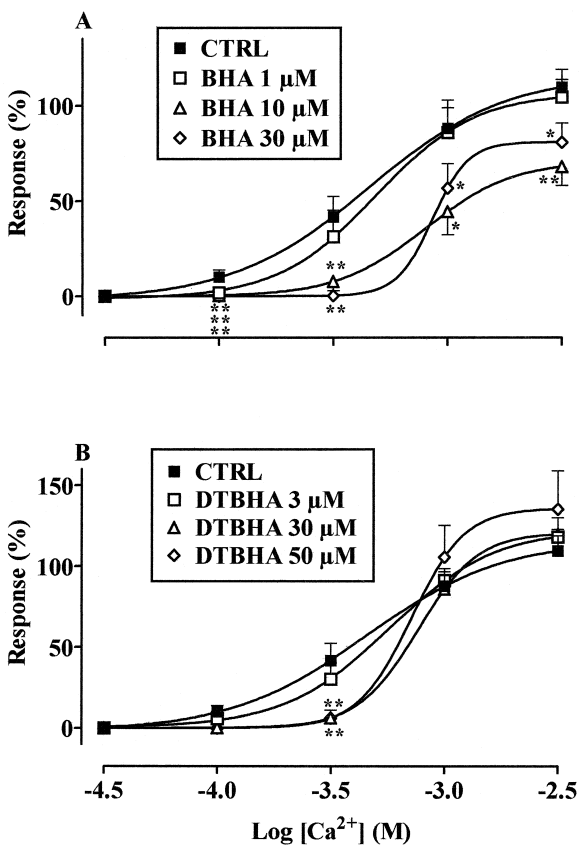

Fig. 5. Relaxant effect of BHA (A) and DTBHA (B) on the response to Ca^{2+} of strips incubated with 10 mM tetraethylammonium. Dimethyl sulfoxide (CTRL), BHA or DTBHA was present for 15 min before and throughout recording of the concentration-effect curves. Cumulative concentration-effect curves for Ca^{2+} were drawn on experimental data normalised against the response to 3 mM Ca^{2+} (taken as 100%) of the initial curve (for further details, see Section 2). Data points are means and vertical lines represent S.E.M. ($n = 4–7$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, * $P < 0.05$ and ** $P < 0.01$ compared to control.

B and C). The addition of 10 μM cyclopiazonic acid elicited a very weak or no contraction (Fig. 4A and B). The subsequent addition of 10 mM tetraethylammonium or 2.5 mM Ba^{2+} led to the appearance of tonic and phasic contractions which were suppressed by addition of 10 μM nifedipine. Similar results were obtained in the absence of cyclopiazonic acid (Fig. 4C) and when the strips were incubated with sodium nitroprusside or isoproterenol instead of BHA (data not shown).

As shown in Fig. 4D, addition of tetraethylammonium increased spontaneous tone and induced phasic contractions. The subsequent addition of BHA suppressed the tetraethylammonium-elicited tonic contraction but not phasic activity. The latter, however, was blocked by nifedipine.

In a second series of experiments, the strips, incubated in normal PSS containing 10 mM tetraethylammonium, developed a tension which averaged 287.0 ± 65.7% ($n = 4$) of the spontaneous tone. BHA antagonised the tetraethylammonium-induced contraction in a concentration-dependent manner, with a pIC_{50} of 6.33 ± 0.16.

In a third series of experiments, the preparations, incubated with Ca^{2+}-free PSS containing 10 mM tetraethylammonium, were stimulated by cumulative concentrations of Ca^{2+} (0.03–3 mM). As shown in Fig. 5A, in the presence of BHA, the concentration-response curve for Ca^{2+} was concentration dependently shifted to the right and the maximum response was depressed, indicating non-competitive inhibition towards Ca^{2+}. DTBHA also concentration dependently antagonised the Ca^{2+}-induced contraction but only up to 0.3 mM Ca^{2+} concentration, leaving the responses elicited by 1 and 3 mM Ca^{2+} unchanged (Fig. 5B). This pattern suggests competitive antagonism towards Ca^{2+}.

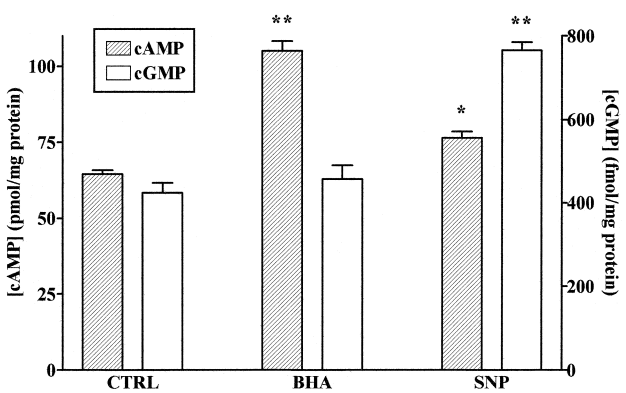

Fig. 6. Effect of BHA and sodium nitroprusside (SNP) on tissue cyclic nucleotide levels. Strips were incubated for 15 min with dimethyl sulfoxide (CTRL), 10 μM BHA or 10 μM sodium nitroprusside. The concentration of cyclic nucleotide was measured radioimmunochemically. Columns are means and vertical lines represent S.E.M. ($n = 4$). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, * $P < 0.05$ and ** $P < 0.01$ compared to control.

3.5. Effects of BHA and sodium nitroprusside on intracellular cyclic nucleotides concentrations

Under standard conditions of incubation, challenge of the strips with 10 μM BHA provoked a significant increase in tissue cAMP concentration ($[cAMP]_i$), but not in cGMP concentration ($[cGMP]_i$) (Fig. 6). In contrasts, challenge with 10 μM sodium nitroprusside markedly increased $[cGMP]_i$ and significantly increased $[cAMP]_i$, though to a lesser extent than with BHA.

4. Discussion

The present results indicate that guinea pig gastric fundus smooth muscle constantly and reliably responds to cyclopiazonic acid with sustained concentration-related contraction, consistent with the observations of Petkov and Boev (1996). The results of experiments with tetrodotoxin and/or atropine also suggest that the effect of cyclopiazonic acid is not neuronal but muscular in nature. Cyclopiazonic acid-promoted depletion of intracellular Ca^{2+} stores may be the signal which activates Ca^{2+} influx from the extracellular space (Fasolato et al., 1994), mainly via the so-called Ca^{2+}-release-activated Ca^{2+} channel (I_{CRAC}), which was first described in mast cells by Hoth and Penner (1992). Ca^{2+} entry dependent on the emptying of intracellular stores (the so called 'capacitative model'; for a review see Berridge, 1995) occurs in several types of cell, if not ubiquitously (Fasolato et al., 1994). In the preparation used here, both spontaneous tone, characterised by a nicardipine-sensitive as well as a nicardipine-resistant component (Duridanova et al., 1995), and cyclopiazonic acid-promoted contraction were dependent on extracellular Ca^{2+}; moreover, cyclopiazonic acid-promoted Ca^{2+} influx was characterised by a nifedipine-resistant component.

BHA not only inhibited cyclopiazonic acid-promoted contraction but also both components of spontaneous tone. At variance, isoproterenol only affected the spontaneous tone, leaving unchanged the cyclopiazonic acid-promoted contraction. The latter effect, however, can be explained by the fact that isoproterenol, owing to its short-lasting action, cannot counteract the effect of cyclopiazonic acid which lasts up to 4 h.

The myorelaxant activity of BHA on rat ileum longitudinal smooth muscle was previously explained on the basis of the blocking of L-type Ca^{2+} channels via its interaction with the dihydropyridine recognition site on the Ca^{2+} channel (Sgaragli et al., 1993). The results of the present study do not sustain this modus operandi, since Bay K 8644 did not overcome BHA-induced relaxation. Furthermore, despite the suppression of spontaneous tone and the impairment of cyclopiazonic acid-elicited tension by BHA, Ba^{2+} and tetraethylammonium still elicited nifedipine-sensitive phasic and tonic contractions on strips incubated with BHA. The same was found when the muscle was

incubated with sodium nitroprusside or isoproterenol. In the absence of cyclopiazonic acid, BHA also completely blocked the tetraethylammonium-induced tonic contraction but not the nifedipine-sensitive phasic component. Indirect inactivation by BHA of L-type Ca^{2+} channels through membrane hyperpolarization seems unlikely, because BHA even relaxes smooth muscle strips contracted with high K^+ concentrations (>50 mM) (not reported here), while myorelaxation induced by K^+ channel openers is abolished by depolarisation (for a review see Quast, 1993). However, at present we cannot rule out the possibility that BHA decreases the sensitivity to Ca^{2+} of contractile elements (Somlyo and Somlyo, 1994; Karaki et al., 1997), especially when considering the increase in $[cAMP]_i$ provoked by BHA in this preparation (see below).

To determine whether there was a relationship between the effects of BHA and the degree of filling of intracellular Ca^{2+} pools, the potent plant alkaloid ryanodine was used. Ryanodine depletes smooth muscle Ca^{2+} stores (Iino et al., 1988) by binding to the Ca^{2+} release channel in the open state, thus producing a long-lasting, sub-conductive open state. The finding that ryanodine failed to counter the BHA reversal of the cyclopiazonic acid-induced contraction suggests either that the effect of BHA is independent of the degree of filling of intracellular Ca^{2+} pools (cyclopiazonic acid- and/or ryanodine-sensitive) or that the ryanodine-sensitive store, which controls cat gastric fundus contractility (Petkov and Boev, 1998), is not a target for BHA.

Significantly, DTBHA, a structural analogue of BHA that possesses an additional *t*-butyl group in the *o*-position relative to the OH group, showed a pharmacological profile which differed from that of BHA. When tested in the contraction model with tetraethylammonium, DTBHA inhibited Ca^{2+} influx competitively and concentration dependently. BHA also inhibited Ca^{2+} influx but in a non-competitive manner. When tested on the cyclopiazonic acid model system, these structurally similar molecules had different effects on the sarcoplasmic reticulum. In fact, DTBHA, a potent inhibitor of the $(Ca^{2+}–Mg^{2+})$-ATPase of skeletal muscle sarcoplasmic reticulum (Sokolove et al., 1986), had no effect, whereas BHA markedly antagonised the effect of cyclopiazonic acid in a competitive manner. In view of these data, the specific effects of BHA and DTBHA can not be ascribed to a general property (e.g., lipophilicity) common to both compounds. Furthermore, since the myorelaxation induced by DTBHA is quite different from that induced by nifedipine, it is unlikely that it depends on the block of voltage-activated Ca^{2+} channels.

Despite the consistent inhibition of muscle contraction by BHA in the present study, once the dihydropyridine recognition site and K^+ channel activation had been excluded, the focus was shifted to an increase in intracellular cyclic nucleotides as the mechanism of action of BHA. BHA, indeed, increased $[cAMP]_i$ but not $[cGMP]_i$, as is the case with isoproterenol (Jin et al., 1993). For lipophilic drugs such as BHA, the partition equilibrium is very much in favour of the plasmalemma lipid bilayer. The intercalation of BHA into cell membranes may alter their microviscosity, leading to substantial changes in their organisation (e.g., lateral phase separations) (Stubbs and Smith, 1984). Adenylyl cyclase is regulated by the nature of its lipid environment, and a decrease in membrane microviscosity has a potentiating effect on its catalytic function and on the synthesis of cAMP (Houslay and Gordon, 1983). The mechanism by which cAMP impairs excitation–contraction coupling in smooth muscle depends on its down-regulating effect on $[Ca^{2+}]_i$ via activation of cAMP-dependent protein kinases. This effect stimulates the sarcoplasmic reticulum Ca^{2+}-ATPase (Raeymaekers and Wuytack, 1993). A direct action of BHA on sarcoplasmic reticulum Ca^{2+}-ATPase can be excluded on the basis of the negative results reported by Moore et al. (1987) for rat liver microsomes and by this laboratory for rat skeletal muscle sarcoplasmic reticulum vesicles (unpublished observation). Once activated, cAMP-dependent protein kinases can stimulate the Na^+/Ca^{2+} exchanger (Blaustein, 1974) as well as Ca^{2+}-activated K^+ channels (Kume et al., 1994), thereby diminishing Ca^{2+} influx across voltage-dependent Ca^{2+} channels. In addition, cAMP-dependent protein kinases can reduce directly Ca^{2+} influx across the plasma membrane (Meisheri and van Breemen, 1982). The net effect of all these processes is a reduced cytosolic Ca^{2+} concentration, which causes relaxation of smooth muscle. Finally, it is known that cyclic nucleotides can cause relaxation via desensitisation of contractile elements to Ca^{2+} (Somlyo and Somlyo, 1994; Karaki et al., 1997). The mechanism by which BHA causes an increase in $[cAMP]_i$ may involve stimulation of adenylyl cyclase activity or an inhibitory effect on a phosphodiesterase (Stryer, 1995). These alternatives are the subject of further study in this laboratory.

Acknowledgements

We are grateful to Prof. E. Masini (Department of Pharmacology, University of Florence, Italy) who measured the cyclic nucleotides. This work was financed by TEMPUS Programme (JEP-0789-92/3) and by Ministero degli Affari Esteri (Rome, Italy) under Law 212 (26-2-1992).

References

Alexandre, A., Doni, M.G., Padoin, E., Deana, R., 1986. Inhibition by antioxidants of agonist evoked cytosolic Ca^{2+} increase, ATP-secretion and aggregation of aspirinated human platelets. Biochem. Biophys. Res. Commun. 139, 509–514.

Berridge, M.J., 1995. Capacitative calcium entry. Biochem. J. 312, 1–11.

Blaustein, M.P., 1974. The interrelationship between sodium and calcium fluxes across cell membranes. Rev. Physiol. Biochem. Pharmacol. 70, 33–82.

Boev, K.K., Bonev, A., Papasova, M., 1985. 4-Aminopyridine-induced changes in the electrical and contractile activities of the gastric smooth muscle. Gen. Physiol. Biophys. 4, 589–595.

Bond, M., Kitazawa, T., Somlyo, A.P., Somlyo, A.V., 1984. Release and recycling of calcium by the sarcoplasmic reticulum in guinea pig portal vein smooth muscle. J. Physiol. 355, 677–695.

Bourreau, J.P., Zhang, Z.D., Low, A.M., Kwan, C.Y., Daniel, E.E., 1991. Ryanodine and the adrenergic, purinergic stimulation in the rat vas deferens smooth muscle: functional and radioligand binding studies. J. Pharmacol. Exp. Ther. 256, 1063–1071.

Duridanova, D.B., Gagov, H.S., Petkov, G.V., Shkodrov, G.B., Boev, K.K., 1995. Cyclic GMP-induced activation of potassium currents by sarcoplasmic reticulum Ca^{2+} pump-dependent mechanism. Gen. Physiol. Biophys. 14, 139–151.

Fasolato, C., Innocenti, B., Pozzan, T., 1994. Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels?. Trends Pharmacol. Sci. 15, 77–83.

Hara, H., Kogure, K., 1990. Prevention of hippocampus neuronal damage in ischemic gerbils by a novel lipid peroxidation inhibitor (quinazoline derivative). J. Pharmacol. Exp. Ther. 255, 906–913.

Harper, J.F., Brooker, G., 1975. Fentomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after two orto acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotide Res. 1, 207–218.

Hoth, M., Penner, R., 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355, 353–356.

Houslay, M.D., Gordon, L.M., 1983. The activity of adenylate cyclase is regulated by the nature of its lipid environment. Curr. Top. Membr. Transp. 18, 179–231.

Iino, M., Kobayashi, T., Endo, M., 1988. Use of ryanodine for functional removal of the Ca^{2+} store in smooth muscle cells of the guinea pig. Biochem. Biophys. Res. Commun. 152, 417–422.

Jin, J.-G., Murthy, K.S., Grider, J.R., Makhlouf, G.M., 1993. Activation of distinct cAMP- and cGMP-dependent pathways by relaxant agents in isolated gastric muscle cells. Am. J. Physiol. 264, G470–G477.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K.-I., Harada, K.-I., Miyamoto, S., Nakazawa, H., Won, K.-J., Sato, K., 1997. Calcium movements, distribution, and function in smooth muscle. Pharmacol. Rev. 49, 157–230.

Kume, H., Hall, I.P., Washabau, R.J., Takagi, K., Kotlikoff, M.I., 1994. β-Adrenergic agonists regulate K_{Ca} channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. J. Clin. Invest. 93, 371–379.

Kuriyama, H., Osa, T., Ito, Y., Suzuki, H., Mishima, K., 1976. Topical differences in excitation and contraction between guinea pig stomach muscles. In: Bülbring, E., Shuba, M.F. (Eds.), Physiology of Smooth Muscle. Raven Press, New York, NY, pp. 185–196.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Meisheri, K.D., van Breemen, C., 1982. Effects of β-adrenergic stimulation on calcium movements in rabbit aortic smooth muscle: relationship with cyclic AMP. J. Physiol. (London) 331, 429–441.

Moore, G.A., McConkey, D.J., Kass, G.E., O'Brien, P.J., Orrenius, S., 1987. 2,5-Di-(*tert*-butyl)-1,4-benzohydroquinone—a novel inhibitor of liver microsomal Ca^{2+} sequestration. FEBS Lett. 224, 331–336.

Petkov, G.V., Boev, K.K., 1996. Cyclopiazonic acid-induced changes in contractile activity of smooth muscle strips isolated from cat and guinea pig stomach. Eur. J. Pharmacol. 318, 109–115.

Petkov, G.V., Boev, K.K., 1998. The role of a ryanodine-sensitive Ca^{2+}-store in the regulation of smooth muscle tone of the cat gastric fundus. Gen. Physiol. Biophys. 17, 225–237.

Putney, Jr., J.W. 1990. Capacitative calcium entry revisited. Cell Calcium 11, 611–624.

Quast, U., 1993. Do the K^+ channel openers relax smooth muscle by opening K^+ channels?. Trends Pharmacol. Sci. 14, 332–337.

Raeymaekers, L., Wuytack, F., 1993. Ca^{2+} pumps in smooth muscle cells. J. Muscle Res. Cell Motil. 14, 141–157.

Ruterbories, K.J., Lindstrom, T.D., 1990. Pharmacokinetics of a novel butylated hydroxytoluene-thiazolidinone CNS anti-ischemic agent LY256548 in rats, mice, dogs and monkeys. Drug Metab. Dispos. 18, 674–679.

Salvemini, D., Masini, E., Pistelli, A., Mannaioni, P.F., Vane, J., 1991. Nitric oxide: a regulatory mediator of mast cell reactivity. J. Cardiovasc. Pharmacol. 17, 258–264.

Seidler, N.W., Jona, I., Vegh, M., Martonosi, A., 1989. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J. Biol. Chem. 264, 17816–17823.

Sgaragli, G.P., Valoti, M., Gorelli, B., Fusi, F., Palmi, M., Mantovani, P., 1993. Calcium antagonist and antiperoxidant properties of some hindered phenols. Br. J. Pharmacol. 110, 369–377.

Shirota, H., Chiba, K., Ono, H., Yamamoto, H., Kobayashi, S., Terato, K., Ikuta, H., Yamatsu, I., Katayama, K., 1987. Pharmacological properties of the novel non-steroidal anti-inflammatory agent *N*-methoxy-3-(3,5-di-*tert*-butyl-4-hydroxybenzylidene)pyrrolidin-2-one. Arzneim-Forsch. 37, 930–936.

Sokolove, P.M., Albuquerque, E.X., Kauffman, F.C., Spande, T.F., Daly, J.W., 1986. Phenolic antioxidants: potent inhibitors of the (Ca^{2+}–Mg^{2+})-ATPase of sarcoplasmic reticulum. FEBS Lett. 203, 121–126.

Somlyo, A.P., Somlyo, A.V., 1994. Signal transduction and regulation in smooth muscle. Nature 372, 231–236.

Steiner, A.L., Parker, C.W., Kipnis, D.M., 1972. Radioimmunoassay for cyclic nucleotides. J. Biol. Chem. 193, 1106–1113.

Stryer, L., 1995. Signal transduction cascades. In: Biochemistry. Freeman, New York, NY, pp. 325–360.

Stubbs, C.D., Smith, A.D., 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim. Biophys. Acta 779, 89–137.

van Breemen, C., Chen, Q., Laher, I., 1995. Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. Trends Pharmacol. Sci. 16, 98–104.

Zoccarato, F., Pandolfo, M., Deana, R., Alexandre, A., 1987. Inhibition by some phenolic antioxidants of Ca^{2+} uptake and neurotransmitter release from brain synaptosomes. Biochem. Biophys. Res. Commun. 146, 603–610.