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Inhibition of prostanoid formation in intact cells by 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone, a blocker of Ca^{2+}-ATPases

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1 The blocker of endoplasmic reticulum Ca^{2+}-ATPase, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) was shown to inhibit formation of prostaglandin E_2 and prostacyclin in the osteoblast-like cell lines, MC3T3-E1 and ROS 17/2.8, respectively, in a dose-dependent manner with an IC_{50} of 0.5–1 μM. Inhibition was observed with various stimuli (arachidonic acid, bradykinin, melittin and calcium ionophore, A23187).

2 This effect was also observed in human platelets, where BHQ dose-dependently blocked thromboxane biosynthesis and formation of 12-hydroxy-heptadecatrienoic acid after stimulation with arachidonic acid, but not formation of 12-hydroxy-eicosatetraenoic acid.

3 Inhibition of prostaglandin E_2 formation in MC3T3-E1 cells was not observed with thapsigargin after stimulation with arachidonic acid, A23187 or melittin, whereas bradykinin-induced prostaglandin E_2 biosynthesis was blocked.

4 Taken together, the results suggest a direct inhibitory action of BHQ on the cyclo-oxygenase in these three cell systems.

Keywords: Cyclo-oxygenase; BHQ; prostaglandins; thromboxane

Introduction

Plasma- and intracellular transmembrane fluxes of Ca^{2+} can be regarded as a major pathway of agonist-response coupling in a variety of cell types (Berridge & Irvine, 1984; Berridge, 1987; Prentki & Matschinsky, 1987). A fundamental process in the signal transduction sequence initiated by the activation of phospholipase C is the transient elevation of $[Ca^{2+}]_i$ by release from intracellular stores, which is thought to be mediated by the generation of cytosolic inositol-(1,4,5)-trisphosphate (IP_3) (Berridge, 1987; Rana & Hokin, 1990). To reduce this primary cell activation and maintain a dynamic, non-exhaustive equilibrium by sequestration of $[Ca^{2+}]_i$, active Ca^{2+} pumps are located in both intracellular and plasma membranes.

Investigations of the molecular mechanisms of cell calcium homeostasis require specific pharmacological tools to modulate Ca^{2+} release from internal stores, and specific inhibitors of the Ca^{2+}-ATPase of endoplasmic reticulum (ER) have been described (Moore *et al.*, 1987; Kass *et al.*, 1989; Thastrup *et al.*, 1990; Bian *et al.*, 1991). Thapsigargin and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ) are widely used because they are membrane permeable and thus can block ER calcium pumps in intact cells (Moore *et al.*, 1987; Kass *et al.*, 1989; Thastrup *et al.*, 1990). Both thapsigargin and BHQ have been shown to exert their inhibitory action by stabilization of the E_2 conformational state of the ATPase (Wictome *et al.*, 1992).

Elevation of $[Ca^{2+}]_i$ may also lead to the activation of phospholipase A_2 (PLA_2) and liberation of arachidonic acid from phospholipids, which is readily metabolized to prostanoids via cyclo-oxygenases in many cell types. It is thus obvious that compounds which modulate levels of $[Ca^{2+}]_i$ might be expected to affect PLA_2 activation and hence prostanoid biosynthesis.

Recently, the proposed blocker of receptor-mediated calcium entry, SK&F 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-imidazole hydrochloride) (Merritt *et al.*, 1990) has been introduced and many of its effects on cellular responses have been related to this inhibitory action. Thus, SK&F 96365 was shown to inhibit isolated sarcoplasmic reticulum Ca^{2+}-ATPase (Mason *et al.*, 1993) as well as prostaglandin formation and receptor-mediated calcium entry in the osteoblast-like cell line, MC3T3-E1 (Leis *et al.*, 1994). Further studies revealed that SK&F 96365 is a potent inhibitor of cyclo-oxygenase in several cell types and that many of the biochemical features attributed to this compound may at least be partly due to inhibition of prostanoid formation (Leis *et al.*, 1995). It was therefore the aim of this study to investigate whether thapsigargin and BHQ might affect the activity of cyclo-oxygenases in different cell types.

Methods

Cell culture

MC3T3-E1 and ROS 17.28 cells were cultured routinely in α-MEM and DMEM, respectively, containing 5% FCS, gentamycin-sulphate (83.4 mg ml^{-1}), and L-glutamine (0.584 g ml^{-1}) in a humidified atmosphere of 5% CO_2 in 80 cm^2 flasks and transferred to 4 cm^2 12-well culture dishes before experiments. At confluency, medium (1 ml) was removed and the cell monolayer incubated in 1 ml of HEPES-buffered Hanks balanced salt solution (HBSS). After preincubation with the indicated concentrations of BHQ or thapsigargin for 15 min, incubations with the appropriate stimuli or vehicle were carried out for 3 min. For prostaglandin measurement, the incubation buffer was removed and processed as described below.

Human platelets were prepared from human plasma as described by Mustard *et al.* (1989) and resuspended in HEPES buffer as described above; 500 μl (10^8 cells) of the suspension were used. Incubation was stopped by addition of 500 μl ice-cold ethanol and thromboxane (TX) B_2 and 12-hydroxy-heptadecatrienoic acid (12-HHT) determined as described below.

Broken cell preparations of washed human platelets were prepared by sonicating the cells 3 times for 5 s at 4°C after addition of compounds to be tested. Incubations with 6 μM

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arachidonic acid or [^{14}C]-arachidonic acid (200000 d.p.m. per sample, 6 μM) were carried out for 30 min. For the non-radioactive experiments, TXB_2 production was determined by GC-MS as described below. Radiolabelled metabolites were extracted and separated into three fractions (AA, 12-HHT + 12-HETE (12-hydroxy-eicosatetraenoic acid), TXB_2) by silicic acid chromatography, as described earlier (Mayer *et al.*, 1986). Radioactivity was counted in a Beckman LSC.

For determination of phospholipase A_2 activity, MC3T3-E1 cells were cultured as described above and prelabelled with [^{14}C]-arachidonic acid (0.1 μCi/well) for 24 h. After removal of the culture medium, the cell layer was washed 3 times with phosphate-buffered saline (PBS) and agonist stimulation was carried out for 30 min in HEPES buffered HBSS. The supernatant was collected and counted for radioactivity in a Beckman LSC.

Prostaglandin determination

PGE_2, TXB_2, 12-HHT and prostacyclin (via its stable metabolite, 6-keto-$PGF_{1\alpha}$) were measured by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) (Mayer *et al.*, 1986; Leis *et al.*, 1987a,b; Malle *et al.*, 1987). Briefly, prostanoids were converted to their PFB ester-TMS-ether-O-methyloxime derivative. Quantitation was carried out by use of tetradeuterated PGE_2, tetradeuterated 6-keto-$PGF_{1\alpha}$ and ^{18}O-TXB_2 as internal standard. 12-HHT was quantified as its PFB ester TMS-ether derivatives after catalytical hydrogenation using $^{18}O_2$-5-HETE as internal standard. A Fisons Trio quadrupole mass spectrometer coupled to a Carlo Erba GC 8000 was used. GC was performed on a 15 m DB5 fused silica capillary column (Fisons Instruments). The temperature of the splitless Grob injector was kept at 290°C, initial column temperature was 160°C for 1 min, followed by an increase of 40°C min^{-1} to 310°C. NICI was carried out in the single ion recording mode with methane as a moderating gas.

Materials

Bradykinin (Bk), melittin (sequencing grade, h.p.l.c. purified, free of phospholipase A_2 activity), arachidonic acid (AA), thapsigargin, BHQ, EGTA, calcium ionophore A23187, PGE_2 and HEPES buffer were from Sigma Chemical Co. (Munich, Germany). DMEM, α-MEM and foetal calf serum (FCS) were obtained from Sera-lab (Vienna, Austria). L-Glutamine was from Serva (Vienna, Austria). Trypsin-EDTA was purchased from Böhringer (Mannheim, Germany). Pentafluorobenzyl bromide (PFBBr), *bis*-(N,O-trimethylsilyl)trifluoroacetamide (BSTFA), silylation grade pyridine, acetonitrile, and O-methoxyamine hydrochloride (MOX) were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Culture dishes were from Falcon via Szabo (Vienna, Austria). [^{14}C]-arachidonic acid was from Amersham, Vienna. MC3T3-E1 and ROS 17/2.8 cells were kindly supplied by Dr Klaushofer, Vienna. Deuterated PGE_2 and 6-keto-$PGF_{1\alpha}$ were obtained through MSD Isotopes via IC Chemikalien GmbH (München, Germany). All other chemicals and reagents were from Merck, Darmstadt, Germany. ^{18}O-TXB_2 and ^{18}O-HETE were prepared as described (Leis *et al.*, 1986).

Results

BHQ inhibited formation of 6-keto-$PGF_{1\alpha}$ in arachidonate-stimulated ROS 17/2.8 osteosarcoma cells (Figure 1) in a dose-dependent manner. IC_{50} values of 1 μM BHQ were obtained. At a drug concentration of 10 μM, 6-keto-$PGF_{1\alpha}$ production was reduced by more than 95% upon treatment with 6 μM arachidonic acid. BHQ at 10 μM also completely inhibited formation of 6-keto-$PGF_{1\alpha}$ after stimulation with the calcium ionophore, A23187 (2 μM) (results not shown). Thapsigargin (100 nM) had no effect on AA- and A23187-induced prostanoid formation in these cells. The effect of BHQ on bradykinin-induced production of 6-keto-$PGF_{1\alpha}$ could not be investigated, since these cells did not show any response to the agonist.

The same inhibitory action was observed for the arachidonic acid- (6 μM) and calcium ionophore, A23187-(2 μM) induced formation of PGE_2 in the clonal murine osteoblast-like cell line, MC3T3-E1. Arachidonate stimulation was completely abolished at 100 μM BHQ (Figure 2). A23187-induced PGE_2 production was inhibited by BHQ with an IC_{50} of 0.3 μM (Figure 3). Again, thapsigargin failed to block AA- and A23187-induced PGE_2 formation. On the other hand, both thapsigargin and BHQ blocked bradykinin-induced biosynthesis of PGE_2 in a dose-dependent manner (Figure 4a and b). No PGE_2 formation was observed with BHQ at 5 μM, whereas thapsigargin above 5 nM reduced prostaglandin levels to basal values, approximately 10% of maximal bradykinin stimulation. Formation of PGE_2 after PLA_2 activation by melittin was also reduced to control levels by BHQ (Figure 2). In no case did addition of BHQ alone induce PGE_2 production.

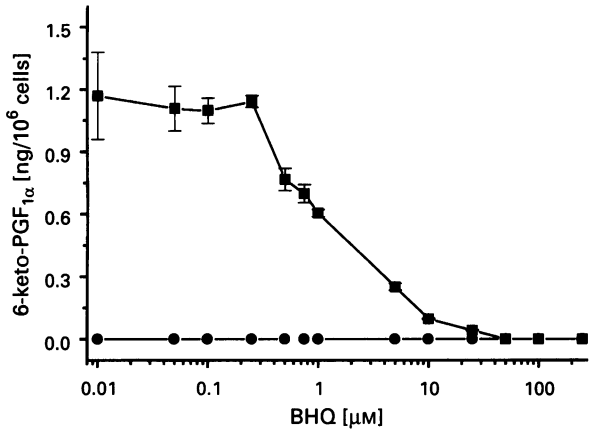

Figure 1 Inhibition of arachidonic acid-induced formation of 6-keto-$PGF_{1\alpha}$ by BHQ in the rat osteosarcoma cell line, ROS 17/2.8. Cells were cultured as described in experimental procedures and preincubated for 15 min with BHQ in HEPES-buffered HBSS. Incubations with arachidonic acid (6 μM) (■) or vehicle alone (●) were subsequently carried out for 30 min. Points represent means ± s.e.mean of 6 determinations.

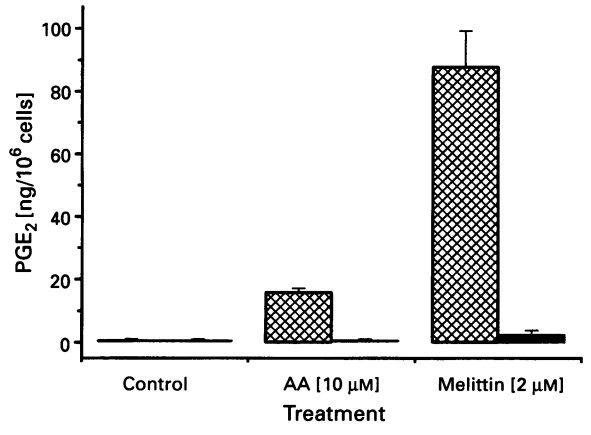

Figure 2 Inhibition of arachidonic acid- and melittin-induced formation of PGE_2 by BHQ in the clonal murine osteoblast-like cell line, MC3T3-E1. Cells were cultured as described in Methods and preincubated for 15 min with BHQ (100 μM) or vehicle. Incubations with arachidonic acid (10 μM) or melittin (2 μM) were subsequently carried out for 30 min. Points represent means ± s.e.mean of 6 determinations.

In arachidonate-challenged human platelets, BHQ blocked synthesis of TXB_2 (Figure 5b). This effect was dose-dependent with an IC_{50} of 10–12 μM. The arachidonic acid concentrations used were 4 μM and 10 μM. No effect of the compound on TXB_2 formation was seen in the absence of arachidonic acid. Additionally, formation of the cyclo-oxygenase-derived metabolite, 12-HHT, was also blocked dose-dependently by the drug (Figure 5a).

The inhibitory effect of BHQ on arachidonate metabolism was also demonstrated directly in broken cell preparations of washed human platelets after stimulation with exogenous arachidonate (6 μM). Without inhibitors, TXB_2 production was 166.6 ± 7.3 ng/10^8 cells. Thapsigargin pretreatment did not alter these values (170.6 ± 9.0 ng/10^8 cells). BHQ (250 μM) reduced thromboxane biosynthesis to 5.6 ± 0.5 ng/10^8 cells, comparable to the inhibitory effect of 5×10^{-7} M indomethacin (7.3 ± 0.1 ng/10^8 cells). Combined addition of BHQ and indomethacin resulted in TXB_2 levels of 6.8 ± 0.1 ng/10^8 cells.

The distribution of radioactivity after incubation of broken human platelets with [^{14}C]-arachidonate is shown in Table 1. The metabolic pattern remained unchanged after thapsigargin pretreatment, whereas BHQ reduced radioactivity in the thromboxane fraction comparable to indomethacin. The bulk of radioactivity (>70%) however is located in the fraction containing the monohydroxylated oxygenation products of arachidonic acid, 12-HHT and 12-HETE. After pretreatment with BHQ, indomethacin or both, a decrease in TXB_2-related radioactivity is paralleled by an increase in this fraction. Under the given experimental conditions the radioactivity measured in fraction II (Table 1) reflects formation of 12-HETE. The radioactivity profile also demonstrates nearly complete conversion of arachidonic acid. Similar results were obtained with broken cell preparations of osteosarcoma cells (results not shown).

Incubations of osteoblast-like cells (MC3T3-E1 and ROS

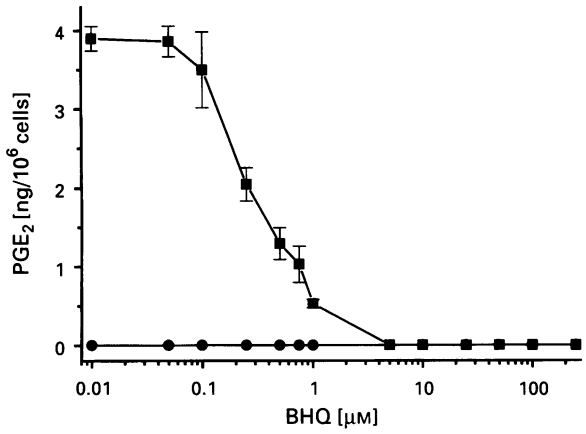

Figure 3 Inhibition of calcium ionophore, A23187-induced formation of PGE_2 by BHQ in the clonal murine osteoblast-like cell line, MC3T3-E1. Cells were cultured as described in Methods and preincubated for 15 min with the indicated concentrations of BHQ. Incubations with A23187 (2 μM) (■) or vehicle alone (●) were subsequently carried out for 30 min. Points represent means ± s.e.mean of 6 determinations.

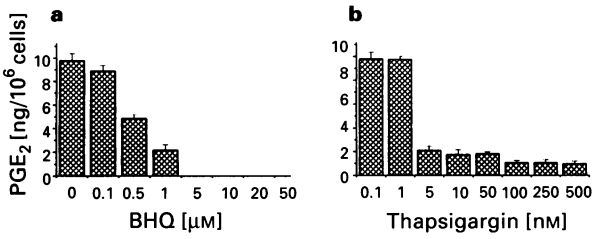

Figure 4 Inhibition of bradykinin-induced formation of PGE_2 by thapsigargin and BHQ in the clonal murine osteoblast-like cell line, MC3T3-E1. Cells were cultured as described in Methods and preincubated for 15 min with the indicated concentrations of BHQ (a) or thapsigargin (b). Incubations with bradykinin (1 μM) were subsequently carried out for 30 min. Points represent means ± s.e.mean of 6 determinations.

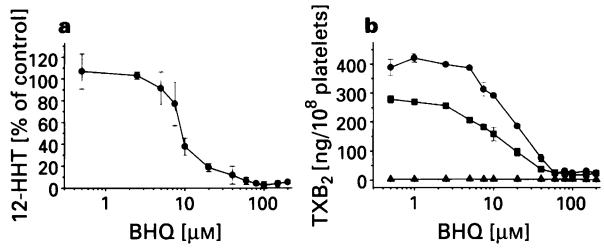

Figure 5 Inhibition of arachidonic acid-induced formation of (a) 12-HHT and (b) TXB_2 by BHQ in washed human platelets. Cell suspensions were obtained as described in Methods and preincubated for 15 min with the indicated concentration of BHQ in HEPES-buffered HBSS, containing 1 mM Ca^{2+}. Incubations with arachidonic acid (10 μM, ●, and 4 μM, ■) or vehicles (▲) were subsequently carried out for 30 min. Points represent means ± s.e.mean of 6 determinations.

Table 1 Fractional distribution of [^{14}C]-arachidonate metabolites in broken human platelets (% of total ± s.e.mean)

	Fraction number		
Pretreatment	I	II	III
None	1.1 ± 0.4	70.9 ± 3.2	28.0 ± 0.9
Thapsigargin	0.7 ± 0.4	69.1 ± 1.1	30.2 ± 1.3
BHQ	1.3 ± 0.5	85.7 ± 2.4	13.0 ± 1.4
Indomethacin	0.8 ± 0.3	87.1 ± 4.9	12.1 ± 1.7
BHQ + indo	1.4 ± 0.4	85.6 ± 3.8	13.0 ± 2.1

Broken cell preparations of washed human platelets were incubated with [^{14}C]-arachidonic acid as described in Methods. Fractions I, II and III were obtained after silicic acid chromatography of the acidic extract according to Mayer *et al.* (1986). *Fraction I:* arachidonic acid and neutral lipids; *fraction II:* mono-hydroxylated metabolites (12-HHT and 12-HETE); *fraction III:* more polar metabolites (TXB_2 and PGs, di-HETES, hepoxilins). The concentrations used were: thapsigargin (100 nM), BHQ (250 μM) and indomethacin (5×10^7 M). Data represent means ± s.e.mean of triplicate determinations.

17/2.8) with [^{14}C]-arachidonic acid, followed by radioscan-thin layer chromatography on Merck Kieselgel G60 plates clearly demonstrated the conversion of the precursor acid to PGE_2 and 6-keto-$PGF_{1\alpha}$, respectively.

Release of radioactivity into the culture medium of [^{14}C]-arachidonate prelabelled MC3T3-E1 cells was measured to assess phospholipase A_2 activity. In untreated controls, the calcium ionophore, ionomycin (2 μM) caused an increase in released radioactivity from 986 ± 117 d.p.m./well to 11920 ± 176 d.p.m./well. With melittin (2 μM), a potent activator of phospholipase A_2, values of 8927 ± 292 d.p.m./well were observed. Pretreatment with BHQ (100 μM) resulted in basal levels of 1231 ± 135 d.p.m./well. Stimulation with ionomycin and melittin produced an increase of released radioactivity to 12723 ± 633 d.p.m./well and 9821 ± 173 d.p.m./well, respectively. Similarly, experiments with thapsigargin (100 nM) pretreatment showed values of 1204 ± 65 d.p.m./well under basal conditions, 11882 ± 41 d.p.m./well after ionomycin challenge and 9231 ± 82 d.p.m./well after melittin stimulation. All values represent means $\pm$ s.e.mean of three determinations.

Discussion

Pharmacological modulation of ER Ca^{2+}-ATPase activity has greatly facilitated investigations on cell calcium homeostasis. Inhibitors like BHQ and thapsigargin have been widely used to probe the molecular mechanisms of intracellular calcium release in intact cells (Moore *et al.*, 1987; Kass *et al.*, 1989; Thastrup *et al.*, 1990; Bian *et al.*, 1991). However, since agonist-induced calcium transients are often found to trigger cellular metabolic responses, such as the formation of autocrine or paracrine modulators like prostanoids, pharmacological side effects could lead to misinterpretation of experimental results. This was recently demonstrated for the proposed blocker of receptor-mediated calcium entry, SK&F 96365, which was shown to be an effective inhibitor of cyclo-oxygenase in several cell types (Leis *et al.*, 1995). Thus, caution should be exercised, when biochemical cellular responses are attributed to the ubiquitous event of calcium influx. Our results clearly demonstrate an inhibitory effect of BHQ on cyclo-oxygenase activity in different cell systems: osteoblast-like cells cloned from 'normal' mouse calvaria, MC3T3-E1 (Kodama *et al.*, 1981), osteoblast-like rat osteosarcoma cells (ROS 17/2.8), and human platelets. Effects of BHQ on phospholipases (e.g. due to blockade of calcium entry) should not be responsible for its inhibitory action on prostanoid formation, since exogenous arachidonic acid would be metabolized in any case. Additionally, stimulation of arachidonate metabolism by the calcium ionophore, A23187, should bypass any effects on phospholipases related to previous depletion of internal calcium stores. Since no such stimulation occurs in MC3T3-E1 and UMR-106 cells upon BHQ pretreatment, the inhibitory effect can be related to the cyclo-oxygenase enzyme. Furthermore, our experiments clearly demonstrate, that phospholipase A_2 activity is not at all affected by pretreatment with BHQ or thapsigargin, as judged by release of radioactivity from [^{14}C]-arachidonate prelabelled MC3T3-E1 cells after stimulation with ionomycin or melittin.

Formation of 12-HHT and TXB_2 in human platelets were also effectively blocked by the drug. To provide further evidence for a cyclo-oxygenase-site of action, experiments were conducted with broken cell preparations of washed human platelets. In this system, BHQ inhibited TXB_2 formation comparable to indomethacin, which was used as an 'inhibitor control'. Fractional separation of the radioactive metabolites revealed, that virtually all of the supplied ^{14}C-labelled arachidonic acid was metabolized, the main fraction being 12-HHT and 12-HETE. Thapsigargin did not alter this pattern, thus confirming a lack of direct inhibitory action on eicosanoid synthesis. Upon treatment with indomethacin or BHQ, however, the prostanoid-associated radioactivity was reduced to a certain basal level. This basal radioactivity could originate from other polar platelet-derived metabolites formed via lipoxygenases, such as hepoxilins and dihydroxylated metabolites, which are also likely to appear in this chromatographic fraction. On the other hand, inhibition of TXB_2 production by BHQ and indomethacin results in an increase of 12-HETE-associated radioactivity in this broken cell preparation. This could be due to enhanced substrate availability for the 12-lipoxygenase enzyme after cyclo-oxygenase blockade.

From these data it is evident, that BHQ is a selective blocker of cyclo-oxygenase, whereas thapsigargin is not. Additionally, the formation of 12-HETE by human platelets is not affected by the drug, thus suggesting a specific inhibitory action without effects on lipoxygenases. On the other hand, both Ca^{2+}-ATPase inhibitors were able to block bradykinin-induced formation of PGE_2 in MC3T3-E1 cells. This is in accordance with the literature, since both BHQ and thapsigargin are thought to empty IP_3-sensitive internal calcium pools (Thastrup *et al.*, 1990), and bradykinin exerts its effect via phospholipase C activation and inositol phosphate breakdown (Berridge, 1987; Moore *et al.*, 1987; Kass *et al.*, 1989; Thastrup *et al.*, 1990; Bian *et al.*, 1991). The release of Ca^{2+} from IP_3-sensitive pools by thapsigargin and BHQ, however, should also be able to activate PLA_2 and hence stimulate prostanoid production to a certain degree (Ohuchi *et al.*, 1989; Malcolm *et al.*, 1992). This was not observed for BHQ, whereas thapsigargin showed dose-dependent reduction of PGE_2 to levels approximately 10% of maximal bradykinin stimulation. This can be explained by the abovementioned stimulation of basal prostaglandin formation by Ca^{2+} release from internal stores by thapsigargin or basal cyclo-oxygenase activity. In the case of BHQ, the inhibitory action on cyclo-oxygenase prevents such basal PGE_2 production.

This indicates care should be used in interpreting experimental data. Nevertheless, the nature of BHQ as a multiple effector ligand provides the possibility of measuring cumulative effects and in combination with specific cyclo-oxygenase inhibitors, of dissociating the two mechanisms of Ca^{2+}-ATPase inhibition and cyclo-oxygenase blockade.

In summary, we conclude that the inhibitor of ER Ca^{2+}-ATPase, BHQ, also blocks cyclo-oxygenase in a dose-dependent manner in several cell types.

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