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Effects of 3,3′-di-*O*-methylquercetin on guinea-pig isolated smooth muscle

SHTAYWY ABDALLA, MUSA ABU ZARGA*, FATMA AFIFI†, SULIEMAN AL-KHALIL†, ADEL MAHASNEH, SALIM SABRI*, *Department of Biological Sciences, *Department of Chemistry, Faculty of Science, †Department of Pharmacognosy, Faculty of Pharmacy, University of Jordan, Amman, Jordan*

Abstract—The effects of the flavone 3,3′-di-*O*-methylquercetin (DOMQ) have been examined and compared with those of quercetin, on guinea-pig isolated ileum, trachea, and main pulmonary artery (MPA). Except for transient contractions induced by low concentrations ($10^{-8}-3\times10^{-6}$ M), DOMQ and quercetin (up to 3×10^{-4}M) caused reduction of the tone and the phasic contractions of the ileum. A23187 reversed the inhibitory effects of quercetin but not those of DOMQ. DOMQ and quercetin caused concentration-dependent relaxation of the trachea and the adrenaline-contracted MPA. DOMQ shifted to the right the concentration-effect curves induced by acetylcholine on the ileum and the trachea, and by adrenaline on MPA and those induced by $CaCl_2$ on ileum, trachea and MPA. DOMQ also inhibited the contractions induced, in Ca^{2+}-free EGTA-containing buffer, by histamine on ileum and by adrenaline on MPA. These observations suggest that DOMQ inhibits Ca^{2+} influx, Ca^{2+} release from intracellular stores and, more likely, Ca^{2+} binding to intracellular receptor proteins.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) has been shown to have inhibitory effects on various enzyme systems. It inhibits the Na^+, K^+-ATPase of the plasma membrane of calf heart, although in a manner different from that caused by ouabain (Kuriki & Racker 1976), the ATPase of bovine heart mitochondria (Lang & Racker 1974) and the Ca^{2+}, Mg^{2+}-ATPase activity of isolated plasma membrane (Gietzen et al 1981). Quercetin also inhibits the sarcoplasmic reticulum Ca^{2+} pump of rabbit skeletal muscle presumably by stabilizing a phosphorylated intermediate of the enzyme (Fewtrell & Gomperts 1977). Many other enzyme systems are also inhibited by quercetin (Bindoli et al 1985; Srivastava 1985). Other effects include inhibition of histamine secretion from rat mast cells, a process that is partially reversed by the Ca^{2+} ionophore A23187 (Fewtrell & Gomperts 1977); it is also a potent reversible inhibitor of the phasic and the tonic contractions of guinea-pig isolated ileum induced by acetylcholine, prostaglandin E_2 and by antigen, suggesting an interference with Ca^{2+} metabolism in that tissue (Macander 1986). As the biological actions of the methylated derivative of quercetin do not appear to have been examined, we hypothesized that the substitution of methoxyl groups for the free hydroxyl groups, would give the methylated derivatives the advantage of stability, since polyphenols are known to be generally susceptible to oxidation of hydroxyl groups by oxygen (Okuda et al 1982). The presence of methoxyl groups at positions 3 and 3′ in DOMQ is especially interesting since it has been suggested that the inhibition of mitochondrial ATPase by quercetin is due to the presence of hydroxyl groups at these two positions (Lang & Racker 1974). We now report on the actions of DOMQ on guinea-pig isolated smooth muscle. Some of the actions of quercetin on these same tissues are also reported for comparison.

Materials and methods

Pairs of lengths of the mid-ileum (2 cm each), the proximal part of the trachea (1 cm each) or the main pulmonary artery (MPA; 5 mm each) were isolated from male albino guinea-pigs (400–600 g) and prepared for recording of isometric contractions using Grass FT03C transducers connected to a Gilson polygraph.

Correspondence to: S. Abdalla, Department of Biological Sciences, Faculty of Science, University of Jordan, Amman, Jordan.

Protocol of the experiments. After 2 h equilibration in the tissue bath, concentration-effect curves of DOMQ and of quercetin on the ileum, trachea and MPA were performed. MPA was precontracted with 3×10^{-6} M adrenaline (to cause 50–70% of maximum contraction). The control tissues were treated with the proper concentration of the solvent of the flavones (0·1 N NaOH in 0·9% NaCl (saline); final concentration of NaOH solution in the tissue bath was 0·9% v/v). In a few experiments with the ileum, when maximum effects were observed, DOMQ or quercetin was washed out to check for the reversibility of their effects. In other experiments when maximum effects were observed, the Ca^{2+} ionophore A23187 was added to the bath to a final concentration of $1\cdot5\times10^{-5}$ M. In all cases, the responses of tissues were expressed as g g^{-1} tissue.

In a second series of experiments, concentration-effect curves to acetylcholine ($10^{-8}-3\times10^{-4}$ M) on the ileum and the trachea, and to adrenaline ($10^{-7}-3\times10^{-4}$ M) on MPA were established in the absence and the presence of 3×10^{-5} M DOMQ added 30 min before the agonist and left in the tissue bath through the experiment. The contractile responses to agonists in the absence and the presence of DOMQ were expressed as g g^{-1} tissue. In a third series of experiments, the physiological salt solution (PSS) was replaced by a nominally Ca^{2+}-free, depolarizing solution in which Na^+ had been replaced isosmotically by K^+. Tissues were incubated in this solution for 30 min then $CaCl_2$ was added cumulatively to a final concentration of 3×10^{-2} M in the absence or presence of 3×10^{-5} M DOMQ. Contractile responses to $CaCl_2$ were expressed as g g^{-1} tissue.

In yet another series of experiments, ileum and MPA were incubated for 25 min in a nominally Ca^{2+} free PSS to which 2 mM EGTA was added 10 min after the addition of the Ca^{2+}-free PSS. Tissues were then incubated in the absence or the presence of various concentration ($10^{-5}-3\times10^{-4}$ M) of DOMQ for 30 min when a single concentration of either 3×10^{-4} M histamine (to the ileum) or 3×10^{-4} M adrenaline (to MPA) was added. The contractile response of each tissue to the agonist in the presence of DOMQ was calculated as a percentage of that in its absence.

Solutions. The composition of PSS in mM was: NaCl 118, KCl 4·7, $CaCl_2\cdot2H_2O$ 2·5, $MgCl_2\cdot6H_2O$ 0·5, NaH_2PO_4 1·0, $NaHCO_3$ 25·0 and dextrose 11·1. The Ca^{2+}-free, depolarizing solution had a similar composition to PSS except that $CaCl_2$ and NaCl were absent and the KCl increased to 122·7 mM. Adrenaline bitartarate (Boehringer Ingelheim) was dissolved in saline and stabilized with 0·05% sodium metabisulphite. Acetylcholine chloride (BDH Chemicals Ltd) and histamine diphosphate (ICN Nutritional Biochemicals) were dissolved in saline. A23187 (Sigma) was dissolved in dimethyl sulphoxide and diluted in distilled water. Quercetin (Carl Roth) and DOMQ, isolated from *Inula viscosa* and identified by direct comparison of its UV, ir, [^{1}H]NMR, MS and m.p. with literature data (Grande et al 1985), were dissolved in a minimal volume of 0·1 M NaOH and the solution made up with 0·9% NaCl. Stock solutions of DOMQ and quercetin were wrapped with aluminium foil to protect against photo-oxidation.

Statistical analyses. Data are presented as means ± s.e.m. The EC50 values of DOMQ on ileum, trachea and MPA were compared with those of quercetin by Student's *t*-test for paired

Table 1. Effects of quercetin and DOMQ on guinea-pig isolated ileum, trachea and main pulmonary artery (MPA)[a].

Treatment	Ileum		Trachea		MPA	
	EC50[b]	Relaxation[c]	EC50	Relaxation	EC50	Relaxation
Quercetin	4·46 ± 0·19	9·6 ± 1·5	5·00 ± 0·71	25·5 ± 1·0	4·01 ± 0·16	36·4 ± 10·7
DOMQ	5·10 ± 0·08[d]	9·8 ± 0·5	5·03 ± 0·53	27·1 ± 1·7	4·03 ± 0·25	145·5 ± 15·2[d]

[a] Values are the mean ± s.e.m. of 6 experiments.
[b] Values are—Log molar EC50.
[c] Values are maximum relaxation expressed in g g^{-1} tissue.
[d] $P < 0·05$ compared with quercetin.

samples after calculation of their log values (Fleming et al 1972). Similarly, the paired *t*-test was used to compare EC50 values of agonists in the absence and the presence of DOMQ, and to compare the responses of tissues at each level of agonist in the absence or the presence of DOMQ. Differences were considered significant when P was $< 0·05$.

Results and discussion

In concentrations from 10^{-7} to 3×10^{-6} M, DOMQ caused mild transient contractions of guinea-pig ileum (maximum contraction of 6·0 ± 0·6 g g^{-1} tissue). Quercetin, in the same concentrations, caused much weaker contractions (1·9 ± 0·5 g g^{-1}). Quercetin has been reported to inhibit Ca^{2+}, Mg^{2+}-ATPase of the plasma membrane (Gietzen et al 1981) and of the sarcoplasmic reticulum (Fewtrell & Gomperts 1977). Such inhibition is expected to lead to an increase of the free cytoplasmic Ca^{2+} and subsequently to a contraction. Methylation of quercetin to give DOMQ, which is assumed to increase the latter's lipid solubility, is expected to potentiate the above effects and thus a larger contraction was obtained with DOMQ than with quercetin. Larger concentrations (10^{-5} to 3×10^{-4} M) of DOMQ and quercetin, caused concentration-dependent inhibition of the phasic contractions and relaxation of the tone of the ileum (Table 1). Judging from the EC50 values, DOMQ was more potent on the ileum than quercetin. The effects of both DOMQ and quercetin were reversible upon their removal from the tissue bath, with DOMQ effects being the more readily reversible. This suggests that these flavonoids do not severely impair the energy production of this tissue. In support of that, it has been shown that certain flavonoids cause only mild respiratory inhibition and uncoupling of oxidative phosphorylation and that these effects have been attributed to the 3′-hydroxyl group (Coleman et al 1984). It is likely that when the 3-and 3′-hydroxyl groups of quercetin are methylated, the above effects on respiration and on phosphorylation are attenuated, hence the more readily reversible effects of DOMQ over those of quercetin. The effects of DOMQ on ileum were not reversed by $1·5 \times 10^{-5}$ M of the Ca^{2+} ionophore A23187 while the phasic contractions of tissues treated with quercetin were restored by it, although not completely. This observation suggests a difference in the mechanism of action of quercetin and DOMQ. The results indicate that while quercetin induces at least some of its effects by inhibiting Ca^{2+} influx, DOMQ seems to be devoid of such activity.

On the trachea, both DOMQ and quercetin ($10^{-7} - 3 \times 10^{-4}$ M) caused concentration-dependent relaxation and both compounds were equally potent (Table 1). Similar relaxation was induced by both compounds on MPA. There was no significant difference between the EC50 values of DOMQ and quercetin on MPA although the maximum relaxation induced by DOMQ was significantly larger than that induced by quercetin (Table 1). On the other hand, DOMQ (3×10^{-5} M) displaced to the right the concentration-effect curves of acetylcholine on the ileum and the trachea and inhibited the maximum contractions induced by 3×10^{-4} M acetylcholine by 80·3 ± 4·2% (n = 6) and 41·4 ± 3·0% (n = 7), respectively. Similarly, DOMQ (3×10^{-5} M) displaced to the right the concentration-effect curve of adrenaline on MPA and inhibited the maximum contractions it induced by 28·8 ± 4·9% (n = 6). Comparison of the EC50 values of acetylcholine on ileum and trachea and adrenaline on MPA in the absence of DOMQ to those in its presence showed that DOMQ increased significantly the EC50 of these agonists (Table 2). The observation that DOMQ displaced to the right the concentration-effect curves of acetylcholine on ileum and trachea, and of adrenaline on MPA suggests that inhibition of smooth muscle contraction by DOMQ is unrelated to the agonist employed. This implies that DOMQ suppresses an agonist-induced biochemical function essential to contraction generation shared by the two agonists used. Similar effects of flavonoids on smooth muscle contraction have been reported (Macander 1986).

To induce contraction of smooth muscle, agonists like acetylcholine and adrenaline are thought to cause an increase of the free intracellular Ca^{2+} by increasing Ca^{2+} influx through receptor-operated or voltage-dependent channels and/or by increasing Ca^{2+} release from cellular depots such as the sacroplasmic reticulum or the mitochondria (Bolton 1979). Agents that inhibit contraction generation on smooth muscle presumably interfere with either influx of, or release of, Ca^{2+} from intracellular stores or by competing with Ca^{2+} for binding proteins such as calmodulin (West 1982). The finding, in the

Table 2. Effects of 3×10^{-5} M DOMQ on the contractions induced by acetylcholine on ileum and trachea or by adrenaline on MPA[a].

Treatment	Ileum		Trachea		MPA	
	EC50[b]	Contraction[c]	EC50	Contraction	EC50	Contraction
Control	6·21 ± 0·12(6)	86·8 ± 9·8(6)	5·41 ± 0·08(7)	62·5 ± 8·7(7)	5·74 ± 0·12(6)	228·0 ± 25·5(6)
DOMQ	< 3·52	14·4 ± 3·2[d](6)	4·57 ± 0·18[d](7)	39·8 ± 5·7[d](7)	5·19 ± 0·07[d](6)	153·8 ± 13·8[d](6)

[a] Values in brackets indicate n.
[b] Values are—Log molar EC50.
[c] Values are maximum contractions induced by agonist and expressed in g g^{-1} tissue.
[d] $P < 0·05$ compared with control.

Table 3. Effects of 3×10^{-5} M DOMQ on $CaCl_2$-induced contractions of guinea-pig ileum, trachea and MPA[a].

Treatment	Ileum		Trachea		MPA	
	EC50[b]	Inhibition[c]	EC50	Inhibition	EC50	Inhibition
Control	$2{\cdot}54 \pm 0{\cdot}05$		$2{\cdot}33 \pm 0{\cdot}15$		$2{\cdot}34 \pm 0{\cdot}14$	
DOMQ	$< 1{\cdot}52$	$73{\cdot}8 \pm 5{\cdot}9$	$1{\cdot}74 \pm 0{\cdot}06$[d]	$19{\cdot}6 \pm 5{\cdot}2$	$2{\cdot}00 \pm 0{\cdot}16$[d]	$40{\cdot}1 \pm 4{\cdot}7$

[a] Values are the mean ± s.e.m. of 6 experiments.
[b] Values are—Log molar EC50.
[c] Expressed as % of control contractions induced by 3×10^{-2} M $CaCl_2$.
[d] $P < 0{\cdot}05$ compared with control.

present study, that DOMQ inhibited agonist-induced contractions suggests that DOMQ interferes with either one or all of the above processes. The exact site of action, however, is not identified by such experiments. To analyse the site of action of DOMQ, we incubated the preparations in nominally Ca^{2+}-free depolarizing solution and added Ca^{2+} exogenously. DOMQ (3×10^{-5} M) displaced to the right the concentration-effect curves of $CaCl_2$ in the ileum, trachea and MPA, and inhibited the contractions induced by 3×10^{-2} M $CaCl_2$ on these tissues (Table 3). In this set of experiments, the source of Ca^{2+} is exclusively extracellular (Godfraind 1981). Inhibition of contraction development by DOMQ in such experiments suggests that DOMQ inhibits Ca^{2+} influx into the smooth muscle cell although this does not rule out the possibility that DOMQ competes with Ca^{2+} for Ca^{2+} binding proteins. If DOMQ inhibits Ca^{2+} influx only, agents like the Ca^{2+} ionophore A23187 would be expected to reverse the inhibition induced by DOMQ. In our experiments A23187 did not restore the phasic contractions nor the tone of ileum treated with DOMQ, while it did so when the ileum was treated with quercetin, suggesting that quercetin inhibited Ca^{2+} influx into smooth muscle cells. This suggestion is consistent with the observation that quercetin inhibits Ca^{2+} influx into ram spermatozoa (Breitbart et al 1985).

To test the possibility that DOMQ affects the release of Ca^{2+} from intracellular stores, tissues were incubated in Ca^{2+}-free, EGTA-containing solution, and exposed to a single large concentration of an agonist (histamine for the ileum and adrenaline for MPA). Contractions induced in these tissues under such conditions are thought to be exclusively due to the release of Ca^{2+} from intracellular stores (Bolton 1979; Brading & Sneddon 1980). Fig. 1 shows that DOMQ inhibited those contractions in a concentration-dependent manner, an observation which suggests that DOMQ either inhibits the agonist-induced release of Ca^{2+} from intracellular stores or that it inhibits the released Ca^{2+} from binding to Ca^{2+} receptor proteins. The results of experiments with A23187 on ileum suggest that the latter possibility is more likely than the former.

Petkov et al (1981) and Nikaido et al (1982) have shown that flavonoids possess an inhibitory action on cyclic (c) AMP phosphodiesterase. Since inhibition of this enzyme is associated with an increase of cAMP, it has been suggested that this increase offers a basis for the explanation of the myolytic activity of flavonoids (Petkov et al 1983). On the other hand, it has been documented that phosphodiesterase, like many other enzyme systems, is regulated by the Ca^{2+}-binding protein calmodulin (West 1982). Our data with Ca^{2+}-free depolarizing solution, Ca^{2+}-free EGTA-containing solution and with Ca^{2+} ionophore A23187, suggest that DOMQ exerts its effects mainly by reversibly binding to Ca^{2+} receptor proteins. It is thus likely that DOMQ binds and consequently antagonizes calmodulin which then regulates many enzyme systems including cAMP phosphodiesterase. Consideration of the consequences of this binding may eventually lead to the myolytic activity of DOMQ described in these experiments.

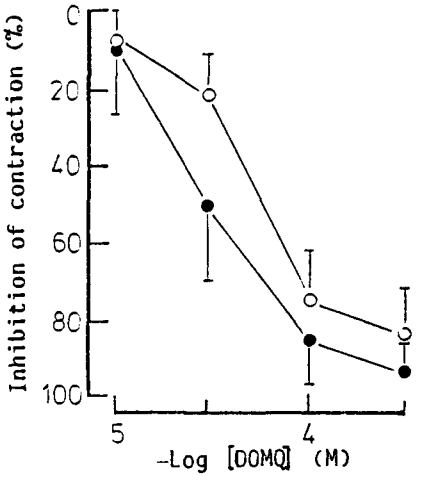

FIG. 1. Inhibition by DOMQ of adrenaline (3×10^{-4} M)-induced contractions of MPA (solid circles) and histamine (3×10^{-4} M)-induced contractions of ileum (open circles) in Ca^{2+}-free, EGTA-containing solution. Vertical bars represent s.e.m.

Finally, our experiments indicate that methylation of the 3- and/or 3'-hydroxyl groups of quercetin induced the following changes in its pharmacologic properties: i) the effects of DOMQ are more readily reversible than those of quercetin, ii) the effects of DOMQ are more resistant to reversal by A23187 than those of quercetin, iii) when used in low concentrations, DOMQ causes contractions of the ileum which are larger than those caused by quercetin. Moreover, methylation of quercetin to DOMQ protected against loss of activity after dissolution. Quercetin solution loses its activity in much shorter time than DOMQ. This loss of activity is expected since polyphenols are more susceptible to oxidation by oxygen (Okuda et al 1982).

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Letters to the Editor

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Ordered powder mixtures: reality or fiction?

H. EGERMANN, *Institute of Pharmaceutical Technology, University of Innsbruck, A-6020 Innsbruck, Innrain 52, Austria*

In 1975, Hersey proposed that the adherence of a finely divided minor ingredient to coarse diluent particles may produce ordered powder mixes of a higher degree of homogeneity than that conforming to the random mix. This concept has become very popular in the past, and even quite recently, ordered mixes were assumed to have been produced (Staniforth 1987).

During the recent years, however, the limitations of ordered mixes have become obvious. From theory, the most serious limitation is the requirement of "ordered adhesion" (Egermann 1980, 1985a). To produce ordered mixes, the mixing operation must accomplish a regular pattern of the adherent fines onto the surface of the carrier component. This assumption is in striking contrast to any evidence available in the fields of particle adhesion and of powder mixing:

(a) In the physics of adhesion, an irregular, random distribution of the adherent particles onto the solids surface is supposed to exist in an equilibrium situation (Krupp 1967; Zimon 1982).

(b) In accordance with this, the many REM-micrographs published of interactive mixes (for refs see Egermann 1984) show an irregular pattern of adherent fines. No micrograph with ordered adhesion could be traced.

(c) Mixing, basically, is a process of disordering. Thus it tends to produce a fully disordered, random distribution of the fines over the total carrier surface in the equilibrium ("random adhesion", Egermann 1980, 1985a).

(d) Adhesion is a process of interaction, not of order (Egermann 1984). To produce ordered mixes, an additional mechanism of order must be available which must enforce the adherent particles to become ordered in the course of the mixing operation. A mechanism of that type has not yet been established.

Without an ordering mechanism being effective, the theoretical best possible mix of interactive constituents is an interactive random mix which shows random adhesion of the fines. The degree of homogeneity of interactive random mixes has been derived from the Poisson-distribution (Egermann 1985b):

$$C_R = 100\ (\bar{m}/G)^{\frac{1}{2}} \qquad (1)$$

C_R is the coefficient of variation of the ingredient content expressed as a percentage of the mean weight G of the ingredient per sample, and $\bar{m}$ is the volume-weighted mean particle weight of the adherent ingredient.

Equation 1 is identical to the equation of Johnson (1972) of the non-interactive random mix as modified by Egermann (1985c). This implies that the highest attainable degree of mixing of interactive powders conforms to that of the non-interactive constituents, and in consequence, that adhesion phenomena cannot produce ordered mixes of higher level of homogeneity under real mixing conditions.

With these theoretical limitations in mind, it is no longer surprising that the existence of ordered mixes still has not been proven by means of experimental evidence. Indeed, some authors, including Hersey (Yip & Hersey 1977) previously claimed to have produced ordered mixes. However, in the light of present knowledge, these claims were a consequence of the erroneous calculation of the quality of the random mix by using the Stange–Poole equation (Stange 1954; Poole et al 1964):

$$C_{Rx} = 100/x\ \{[xy(ym_x + xm_y)]/M\}^{\frac{1}{2}} \qquad (2)$$

C_{Rx} is the coefficient of variation as a percentage of the mean drug content per sample of constant mass M, x and y are the mean proportions of the drug and the diluent per sample, $\bar{m}_x$ and $\bar{m}_y$ are the mean particle masses of the drug and the diluent.

Equation 2, though widely used, applies to constituents of similar particle size only (Stange 1954; Sommer 1976), a fact which has been overlooked in the past. If the diluent component is large in particle size compared with the drug particle size, equation 2 yields too high values of C_{Rx}, as has been verified