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Effects of simple aromatic compounds and flavonoids on Ca^{2+} fluxes in rat pituitary GH_4C_1 cells

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Abstract

The biological activity of phenolic compounds from plants is well documented in vitro, but little is known about the possible effect of simple aromatic compounds and flavonoids on voltage-operated Ca^{2+} channels (VOCCs). In pituitary cells, several intracellular pathways may regulate the activity of VOCCs. In this study, we investigated the effect of nine phenylpropanes and metanes, and 20 flavonoids on high K^+-induced $^{45}Ca^{2+}$ entry in clonal rat pituitary GH_4C_1 cells. At the highest dose tested (20 μg/ml), flavone (a flavone) inhibited $^{45}Ca^{2+}$ entry by 63.5%, naringenin (a flavanone) by 56.3% and genistein (an isoflavone) by 54.6%. The phenylmetane derivative octyl gallate was the most potent compound tested, with an IC_{50} value of 15.0 μg/ml. The IC_{50} value for the reference compound verapamil hydrochloride was 3.0 μg/ml. In sharp contrast to the above, the flavonols quercetin and morin potentiated $^{45}Ca^{2+}$ entry. At 20 μg/ml, quercetin increased $^{45}Ca^{2+}$ entry by 54.1% and morin by 48.0%. Quercetin increased the cellular cAMP content in a concentration-dependent manner. H 89, an inhibitor of protein kinase A, inhibited the effect of quercetin on $^{45}Ca^{2+}$ entry. The results thus suggest that the effect of quercetin is the result of a protein kinase A-mediated activation of VOCCs. Quercetin induced a rapid and marked increase in both the transient (143.1 ± 4.2%) and delayed (198.8 ± 10.0%) Ca^{2+} currents, measured by the whole cell patch clamp technique. The onset of the inhibitory effect of octyl gallate was slow, but resulted in an almost complete inhibition of both Ca^{2+} currents.

Keywords: Ca^{2+} current; Flavonoid; Pituitary GH_4C_1 cell, rat; Voltage clamp, whole cell; VOCC, Voltage operating Ca^{2+} channel

1. Introduction

Only plants and microorganisms are capable of biologically synthesizing the aromatic nucleus, which is a base structure in plant phenolic compounds produced via the shikimic acid pathway. For example, flavonoids are polyphenolic substances that are based on a flavan nucleus consisting of 15 carbon atoms arranged in three rings (C6–C3–C6, see Fig. 1). The effects of flavonols and flavones on the enzymes involved in the regulation of cell division and proliferation, platelet aggregation, detoxification, as well as inflammatory and immune responses have been described (Middleton and Kandaswami, 1994). Phenolic compounds have been reported to interfere with various stages of cancer development (Stavric, 1995; Huang and Ferraro, 1992), and a number of naturally occurring as well as synthetic flavonoids have been shown to have potent anti-human immunodeficiency virus (HIV) activity in vitro (Wang et al., 1998). Because of their phytoestrogenic properties, flavonoids, as well as other plant phenolic compounds, have also been implicated in preventing menopausal symptoms, osteoporosis, breast and ovarian cancer, as well as heart disease (Adlerkreutz et al., 1992; Kurzer and Xu, 1997). In vivo studies suggest that espe-

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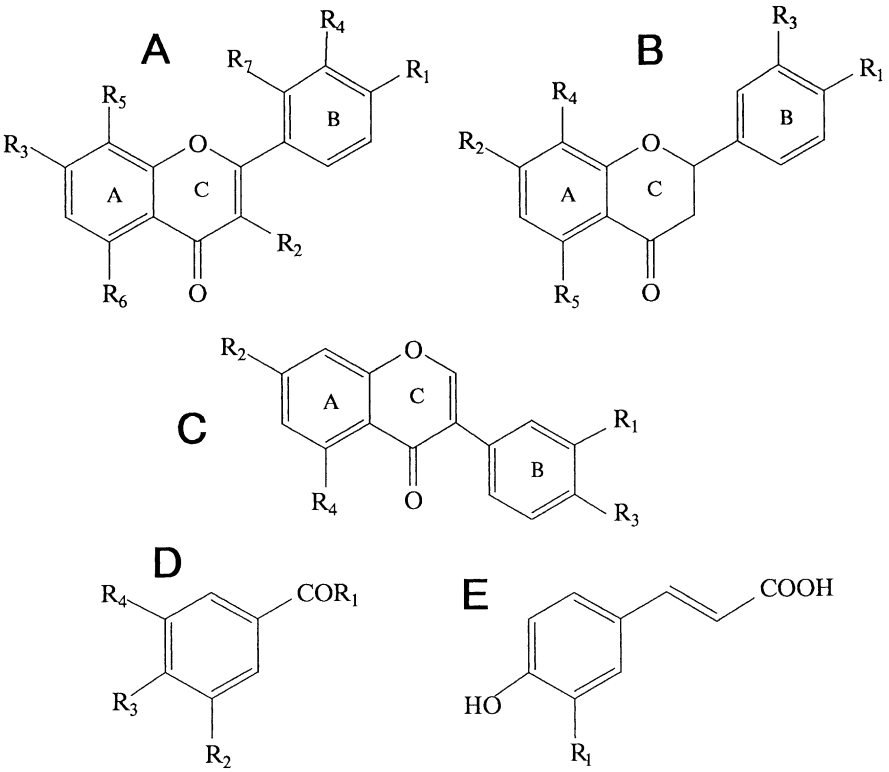

Fig. 1. Basic structures of the phenolic compounds tested on Ca^{2+} fluxes in clonal rat pituitary CH_4C_1 cells: (A) flavones and flavonols, (B) flavanones, (C) isoflavones, (D) phenylmetanes, and (E) phenylpropenes. Substituents R_{1-7} relate to the corresponding functional groups listed in Table 1.

cially flavonoids may reduce the risk of coronary disease (Fotsis et al., 1993; Kapiotis et al., 1997). In fact, epidemiological studies indicate a beneficial role of flavonoids in diminishing the risk of coronary heart disease (Hertog et al., 1993; Hollman et al., 1996).

The effects of flavonoids on the cardiovascular system has been attributed to a modulation of Ca^{2+} homeostasis. The antagonistic activity of flavonoids and other plant phenolic compounds has usually been investigated by measuring the inhibition of depolarization-induced contractions of smooth muscle preparations (Vuorela et al., 1997). These contractions are apparently evoked by Ca^{2+}-dependent mechanisms and are indeed inhibited by Ca^{2+} channel antagonists (Hof and Vuorela, 1983). The L-type voltage-operating Ca^{2+} channels (VOCCs, i.e. slowly inactivating VOCCs) are of crucial importance in the regulation of excitation–contraction coupling in cardiac and vascular smooth muscle (Tsien and Tsien, 1990). Several flavonoids modulate protein tyrosine kinase, protein kinase A and C dependent pathways in different cell systems (Ferriola et al., 1989; Duarte et al., 1993a; Agullo et al., 1997; Revuelta et al., 1997). Inhibition of protein tyrosine kinase by genistein has been shown to inhibit voltage-gated potassium channels (Smirnov and Aaronson, 1995), and retinal cyclic nucleotide-gated channels are inhibited as a result of inhibition of protein tyrosine kinase (Mergler et al., 1998). In pituitary cells, several intracellular pathways (e.g., protein kinase A, protein kinase C and protein tyrosine kinase) modulate the activity of the VOCCs, and thus a multitude of Ca^{2+}-dependent physiological processes, e.g. hormone secretion and synthesis (Tan and Tashjian, 1984; Albert and Tashjian, 1984; Cataldi et al., 1996).

The antagonistic activity of certain plant phenolic compounds has long been acknowledged. The previous works have usually been focused on depolarization-induced contraction in smooth muscle preparations. The effects have been reported to be related to Ca^{2+} fluxes or metabolism. VOCCs can be seen as a target of intracellular pathways, e.g. protein kinase A, protein kinase C and protein tyrosine kinase. The effects of plant phenolic compounds suggest that these compounds may regulate Ca^{2+} fluxes in several endocrine glands. Therefore, the aim of the present study was to screen the effects of simple aromatic compounds and different classes of flavonoids on high K^+-evoked entry of $^{45}Ca^{2+}$ in well-characterised clonal rat GH_4C_1 pituitary cells.

2. Materials and methods

2.1. Materials

The culture medium, serum, lanthanium chloride, and penicillin–streptomycin used for cell culture were purchased from Gibco BRL (UK) and Sigma (MO, USA). Dulbecco's phosphate-buffered saline (PBS) was from

Gibco BRL, and EDTA from Sigma. Falcon tissue culture dishes of Ø 35 mm (3001) and Ø 100 mm (3003) were obtained from Becton Dickinson (UK). All other chemicals were of reagent grade. $^{45}CaCl_2$ (2.0 mCi/ml) was purchased from Pharmacia Amersham Biotech (UK), and Optiphase Hisafe 2 liquid scintillation solution from Fisons Chemicals (UK). H-89, (*N*-[2-(*p*-bromocin namylamino) ethyl]-5-isoquinolinesulfonamidedihydrochloride) was purchased from Calbiochem-Novabiochem (CA, USA). Verapamil hydrochloride was obtained from Orion Pharmaceuticals (Finland). The structures and sources of the simple phenolic compounds and flavonoids are presented in Table 1 and Fig. 1. All test compounds were dissolved in dimethylsulphoxide (DMSO, Merck, Germany).

2.2. Cell culture

The clonal rat pituitary GH_4C_1 cells were originally a gift from Dr. A.H. Tashjian, Jr. (Harvard University, Boston, MA, USA). GH_4C_1 cells were cultivated (according to Tashjian, 1979) in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum ($F\text{-}10^+$) and penicillin–streptomycin (50 + 50 IU/ml) in a water-saturated atmosphere of 5% CO_2 and 95% air, at 37°C.

Table 1
Structures of investigated compounds and their effect on $^{45}Ca^{2+}$ uptake (%). All values are the mean ± S.E.M. of triplicate determinations

Compound (20 μg/ml)	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Effect on Ca^{2+} uptake	S.E.M.	M_w
Flavones (structure A)										
Apigenin[a]	OH	H	OH	H	H	OH	H	**−29.3**	4.0	270.2
Luteolin[a]	OH	H	OH	OH	H	OH	H	**−51.4**	7.7	286.2
Acacetin[a]	OCH_3	H	OH	H	H	OH	H	**−1.39**	1.9	284.3
Flavone[a]	H	H	H	H	H	H	H	**−63.5**	3.0	222.2
Vitexin[a]	OH	H	OH	H	Glu	OH	H	**−2.12**	5.1	432.4
Vitexin-2″-*O*-rhamnoside[a]	OH	H	OH	H	GluRha	OH	H	**−14.6**	0.3	587.5
Luteolin-7-glucoside[a]	OH	H	OGlu	OH	H	OH	H	**−16.3**	1.3	448.4
Luteolin-3′,7-glucoside[a]	OH	H	OGlu	Oglu	H	OH	H	**−14.6**	3.3	610.5
Flavonols (structure A)										
Quercetin[a]	OH	OH	OH	OH	H	OH	H	**+54.1**	6.9	302.2
Rhamnetin[a]	OH	OH	OCH_3	OH	H	OH	H	**+6.63**	0.8	316.3
Isorhamnetin[a]	OH	OH	OH	OCH_3	H	OH	H	**+52.4**	2.3	316.3
Morin[a]	OH	OH	OH	H	H	OH	OH	**+48.0**	5.9	302.2
Quercitrin[a]	OH	ORha	OH	OH	H	OH	H	**+20.1**	0.6	448.4
Rutin[a]	OH	ORut	OH	OH	H	OH	H	**−3.88**	6.0	610.5
Flavanones (structure B)										
Naringenin[a]	OH	OH	H	H	OH	–	–	**−56.3**	5.6	272.3
Naringin[a]	OH	ORhaGlu	H	H	OH	–	–	**+6.5**	7.5	580.5
Isoflavones (structure C)										
Daidzein[a]	H	OH	OH	H	–	–	–	**−26.2**	1.2	254.2
Genistein[a]	H	OH	OH	OH	–	–	–	**−54.6**	1.7	270.2
Daidzin[a]	H	OGlu	OH	H	–	–	–	**+7.6**	5.9	416.4
Genistin[a]	H	OGlu	OH	OH	–	–	–	**−3.39**	5.9	432.4
Phenylmetanes (structure D)										
Benzoic acid[b]	OH	H	H	H	–	–	–	**−9.82**	1.9	122.1
Gallic acid[b]	OH	OH	OH	OH	–	–	–	**−5.33**	1.3	170.1
Syringic acid[a]	OH	OCH_3	OH	OCH_3	–	–	–	**−10.9**	4.0	198.2
Methyl gallate[c]	OCH_3	OH	OH	OH	–	–	–	**−21.2**	6.4	184.1
Propyl gallate[d]	$O(CH_2)_2CH_3$	OH	OH	OH	–	–	–	**−37.9**	4.1	212.2
Octyl gallate[d]	$O(CH_2)_7CH_3$	OH	OH	OH	–	–	–	**−92.2**	7.8	282.3
Dodecyl gallate[d]	$O(CH_2)_{11}CH_3$	OH	OH	OH	–	–	–	**−40.4**	6.8	338.4
Phenylpropenes (structure E)										
Caffeic acid[a]	OH	–	–	–	–	–	–	**+9.71**	2.4	180.2
Ferulic acid[c]	OCH_3	–	–	–	–	–	–	**+9.23**	2.1	194.2

M_w: Molecular weight of the compound (g/mol).
Glu = Glucose, Oglu = OGlucose, ORha = ORhamnose , ORut = ORutinose, OGluRha = OGlucoseRhamnose, ORhaGlu = OrhamnoseGlucose.
[a]Source: Roth (Germany).
[b]Source: Merck (Germany).
[c]Source: Sigma (MO, USA).
[d]Source: Fluka (Switzerland).

Before an experiment, the cells from a single donor culture were harvested with 0.02% EDTA in PBS solution and plated onto Ø 35-mm culture dishes. The cells were cultured for 7 days in Ham's F-10^+ medium before each test, with three changes of the culture medium. Fresh medium was always added 24 h prior to an experiment.

2.3. Measurement of $^{45}Ca^{2+}$ uptake in clonal rat pituitary GH_4C_1 cells

$^{45}Ca^{2+}$ uptake via VOCC was measured as described previously (Tan and Tashjian, 1984). The medium was aspirated from the cultures and the cells were preincubated in 1 ml buffered salt solution (BSS; in millimolar concentrations: NaCl, 130.6; KCl, 5.9; $CaCl_2$, 0.4, $MgCl_2$, 1.2; glucose, 11.8; HEPES, 18.0; pH 7.3) (Härmälä et al., 1992) at 37°C for 10 min. After preincubation, the BSS was aspirated, and 1 ml BBS containing 50 mM K^+, $^{45}Ca^{2+}$ (0.16 mCi/ml) and the test compound was added to the dishes. The incubation was then continued at 37°C for 15 min. In the control experiment, the cells were incubated with BSS containing 50 mM K^+, $^{45}Ca^{2+}$ (0.16 mCi/ml) and the vehicle. The effect of the test compounds on basal uptake of $^{45}Ca^{2+}$ was tested by incubating the cells with normal BSS containing $^{45}Ca^{2+}$ and the test compounds. In some experiments the cells were incubated with the protein kinase A-inhibitor H-89 (10 μM), 50 mM K^+, $^{45}Ca^{2+}$ and the test compound for 20 min. At the end of the incubation period, the medium was aspirated and the cells were washed three times with BSS buffer containing La^{3+} and no $^{45}Ca^{2+}$. The cells were solubilized with 0.1 N NaOH, and the cell-associated radioactivity was measured by liquid scintillation counting (Wallac Winspectral 1414 Liquid Scintillation Counter, Wallac, Finland). Verapamil hydrochloride dissolved in DMSO was used as a reference compound.

2.4. Measurement of cellular cAMP production

GH_4C_1 cells were grown in Ham's F10^+ medium for 5–7 days. The cells were harvested by incubating them in PBS containing 0.02% EDTA. The cells were washed twice with BSS, resuspended in BSS, and aliquots were added to test tubes containing quercetin (0.2–20 μg/ml). The cells were incubated in a water bath at 37°C for 20 min. The incubation was terminated by the addition of perchloric acid to a final concentration of 0.1 M. The samples were neutralized with KOH, and after a 10-min centrifugation at $720 \times g$ the cAMP content in the supernatant was determined with a protein-binding assay as described previously (Norstedt and Fredholm, 1990), with the exception that the bound cAMP was separated from free cAMP using a Millipore MultiScreen filtration system and glass fiber type B opaque filter plates (Millipore, Finland). The filters were washed three times with 250 μl of 50 mM Tris buffer (pH 7.5), dried and then 50 μl SuperMix scintillation fluid (Wallac) was added and the radioactivity was counted using a Wallac MicroBeta counter (Wallac).

2.5. The whole-cell patch-clamp technique

Voltage-clamp experiments were conducted using the whole-cell patch-clamp technique with an EPC9 amplifier and Pulse and Pulsefit software (Heka, Germany). The patch pipettes had a resistance of 3–5 MW when filled with intracellular solution containing (in mM) CsCl, 120; MgATP, 5; *O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 10 and HEPES, 10 (pH 7.2). The cells were continuously superfused with high $^{45}Ca^{2+}$ BSS (BSS supplemented with 9 mM $CaCl_2$), and quercetin or octyl gallate (20 μg/ml) was added directly to the solutions from stocks made in DMSO.

2.6. Calculation of IC_{50} values and statistics

$^{45}Ca^{2+}$ uptake was measured in triplicate and repeated six times. The concentration giving 50% inhibition (IC_{50}) was determined for the tested compounds by linear regression analysis of curves obtained for three doses (0.2, 2.0 and 20 μg/ml; two separate determinations with triplicate dishes), based on the idea of low, medium and high doses used for bioassays in the European Pharmacopoeia (1997). Statview + Graphics® for Macintosh was used for the calculations.

3. Results

3.1. Inhibitory effect on $^{45}Ca^{2+}$ uptake

Recent studies have suggested that isoflavonoids may inhibit VOCCs (Wijetunge et al., 1992; Wijetunge and Hughes, 1995). We found that several phenolic compounds had a clear Ca^{2+} antagonistic activity similar to that of the therapeutically used Ca^{2+} channel antagonist verapamil hydrochloride. The flavone aglycones luteolin and flavone produced a 51.4% and a 63.5% inhibition at the highest tested concentration (20 μg/ml). Flavones with sugar moieties had the weakest inhibitory effect on $^{45}Ca^{2+}$ uptake. The flavanone aglycone naringenin produced 56.3% inhibition at 20 μg/ml. The same compound with a sugar moiety in the R_2 position had negligible activity at the highest concentration tested (20 μg/ml). The isoflavone genistein inhibited high K^+-evoked $^{45}Ca^{2+}$ entry by 54.6% at 20 μg/ml, while genistin (R_2 = glucose) was without effect. A similar result was obtained when daidzein and daidzin were tested, although the effect of daidzein was weaker than that of genistein (Table 1).

The phenylmetane derivatives dodecyl gallate and octyl gallate were the most effective Ca^{2+} channel antagonists of the tested phenylmetanes, showing a 40.4% and a

92.2% inhibition at 20 μg/ml, respectively. The phenylmetanes benzoic, gallic and syringic acid as well as the phenylpropenes caffeic and ferulic acid had only marginal effects on the K^+-evoked uptake of $^{45}Ca^{2+}$ (Table 1). A concentration–response curve was made for octyl gallate, and from this curve an IC_{50} value of 15.0 μg/ml was obtained. The IC_{50} value obtained for verapamil was 3.0 μg/ml. Thus, the effect of octyl gallate was very similar to that of verapamil, making it a potent inhibitor of Ca^{2+} entry through VOCCs.

3.2. $^{45}Ca^{2+}$ entry activators

A most surprising observation was that some of the flavonoids and simple phenolic compounds appeared to be potent activators of Ca^{2+} entry (Table 1). The flavonoids morin, isorhamnetin and quercetin all enhanced the K^+-evoked uptake of $^{45}Ca^{2+}$ by 48.0%, 52.4% and 54.1%, respectively, at a concentration of 20 μg/ml. The flavonolglycoside, quercitrin, had a modest effect, while rutin and rhamnetin were without an effect on Ca^{2+} entry. Concentration–response experiments were conducted also for morin and quercetin, using the concentrations 0.4, 4.0 and 40.0 and 120 μg/ml. In these experiments, higher concentrations were used in order to see whether the enhancing effect would turn into an inhibitory effect. However, even at the highest dose tested (120 μg/ml), both morin and quercetin still enhanced Ca^{2+} entry (Fig. 2).

The above results led us to test whether quercetin, isorhamnetin, and morin per se would increase $^{45}Ca^{2+}$ entry, without prior K^+ depolarization. In this experiment quercetin still increased $^{45}Ca^{2+}$ entry by 43.0%. Isorhamnetin and morin increased Ca^{2+} entry by 13.0% under these conditions. This effect is probably due to the spontaneous membrane potential oscillations in these cells, which result in activation of VOCCs even in the absence of K^+ stimulation (Tsien and Tsien, 1990). A possible mechanism for the effects of quercetin, isorhamnetin, and morin on Ca^{2+} entry could be via cAMP, as quercetin has been previously shown to increase cAMP in human platelets and to prevent their aggregation (Lanza et al., 1987). cAMP activates protein kinase A, which in GH_4C_1 cells upmodulates VOCCs (Törnquist, unpublished results). To test this hypothesis, we measured the effects of quercetin on cAMP levels in GH_4C_1 cells. As seen in Fig. 3A, quercetin induced an increase in cAMP at concentrations which increased $^{45}Ca^{2+}$ entry. Furthermore, the protein kinase A inhibitor H-89 abolished the quercetin-induced increase in $^{45}Ca^{2+}$ entry (Fig. 3B), strengthening the conclusion that the enhanced entry of $^{45}Ca^{2+}$ evoked by quercetin is the result of an increase in cAMP and a concomitant activation of protein kinase A leading to upmodulation of the VOCCs.

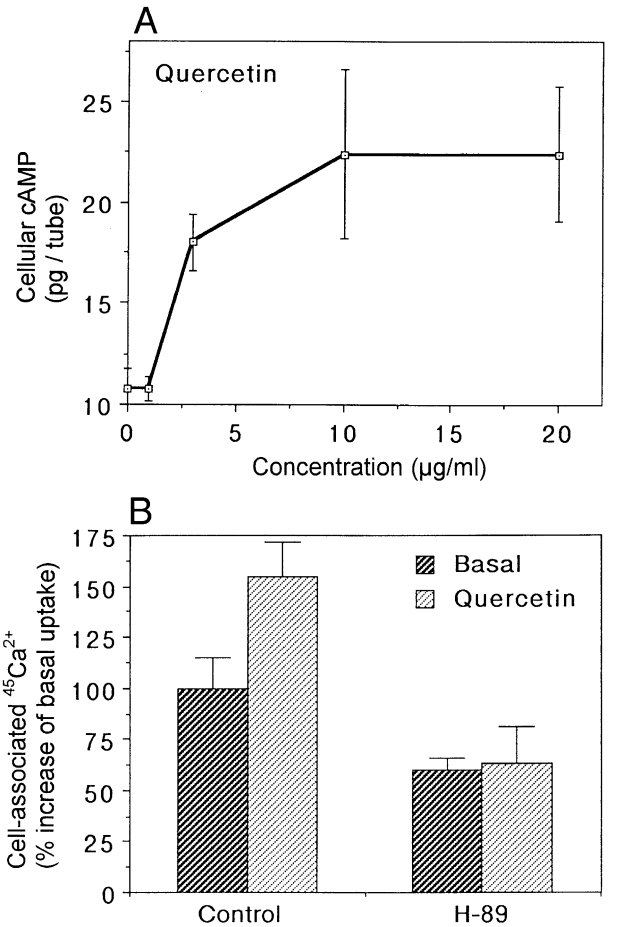

Fig. 3. Effect of quercetin on cellular cAMP and Ca^{2+} entry in CH_4C_1 cells. (A) Aliquots of cells were incubated with four concentrations of quercetin (1.0, 3.0, 10.0 and 20.0 μg/ml) and the cellular cAMP content was measured as described in Materials and methods. (B) The cells were pretreated with H-89 (final concentration 10 μM) or with vehicle (Control), and the high K^+ evoked entry of $^{45}Ca^{2+}$ was measured. The points given are the means ± S.E.M. of triplicate dishes.

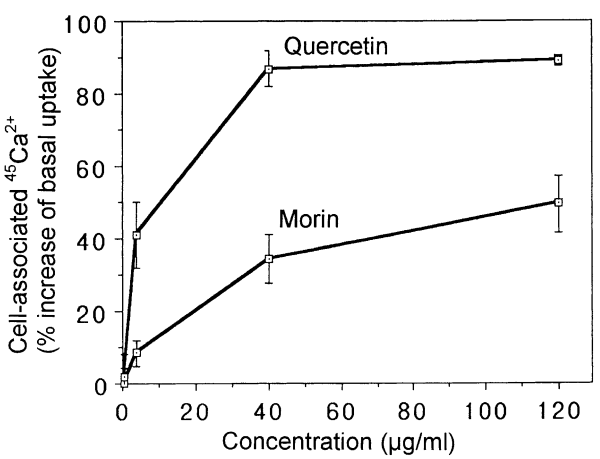

Fig. 2. The activating effect of morin and quercetin on Ca^{2+} entry in rat pituitary CH_4C_1 cells. The cells were stimulated with the indicated concentrations of morin and quercetin, and the uptake of $^{45}Ca^{2+}$ was measured. The points given are the means ± S.E.M. of triplicate dishes.

3.3. The effect of quercetin and octyl gallate on VOCC-mediated membrane currents

The marked increase in $^{45}Ca^{2+}$ entry observed with quercetin prompted us to take a closer look at its action on VOCCs by means of the whole-cell patch-clamp technique. As evident from Fig. 4B, quercetin (20 μg/ml) induced a marked potentiation of the maximal Ca^{2+} current observed upon depolarisation from a holding potential ranging from −70 up to −10 to +10 mV. Quercetin also induced a slight shift in the current–voltage relationship of the VOCCs. In the control solution, the Ca^{2+} current peaked at around 0 mV, whereas in the presence of quercetin the maximum current was observed between −10 and 0 mV. The mean quercetin-induced maximal transient current observed in experiments similar to that shown in Fig. 4C and D was 143.1 ± 4.2%, and that of the late, delayed current 198.8 ± 10.0% ($n = 5$). Fig. 4E shows the time course of the action of quercetin on the VOCCs. The onset of the quercetin effect was fairly rapid, peaking in about 10 s after the onset of quercetin perfusion. Despite the pronounced rundown of $^{45}Ca^{2+}$ currents typical of the experimental conditions used (Titievsky et al., 1998), the effect of quercetin was always at least partially reversible.

We also conducted similar experiments with octyl gallate, which had a potent inhibitory effect on $^{45}Ca^{2+}$ entry, as shown above. As expected, octyl gallate (20 μg/ml) produced almost complete inhibition of the VOCCs for the transient (−73.8 ± 12.2%) and delayed (−84.4 ± 14.2%) currents, respectively (values are mean of all measurements of the current increase, $n = 4$, Fig. 5). It had a clearly slower onset of action than quercetin (data not

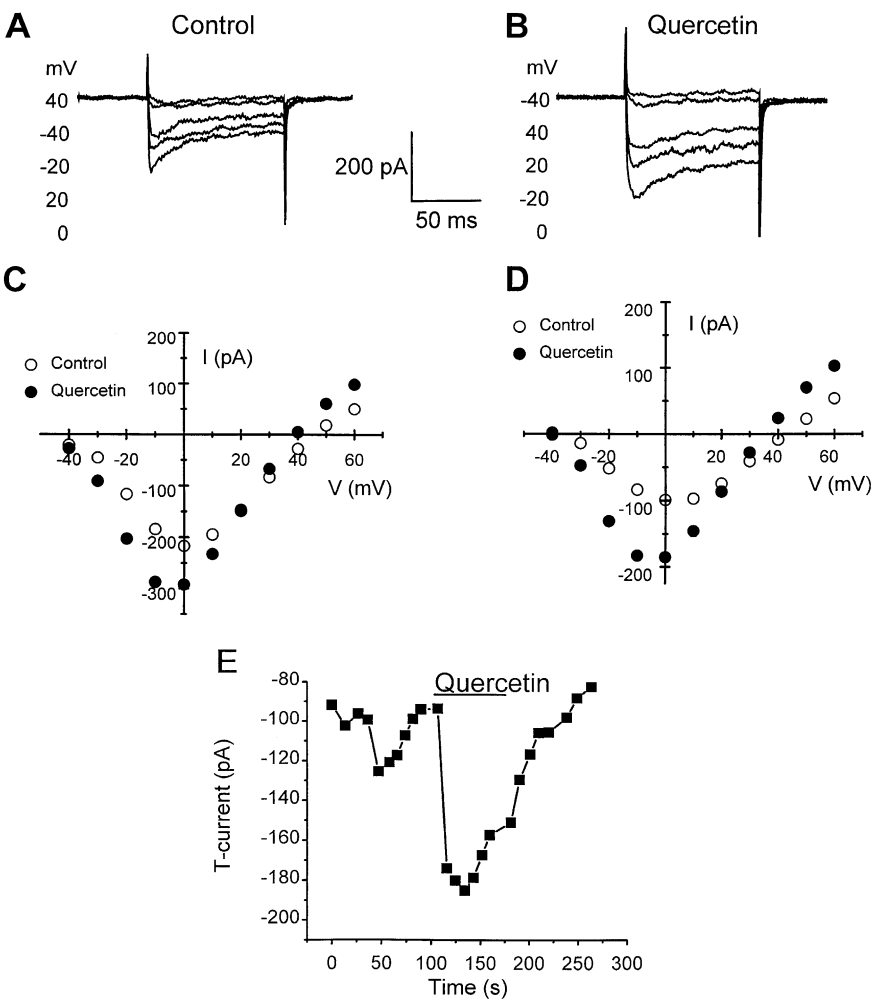

Fig. 4. Effect of quercetin on Ca^{2+} currents in a CH_4C_1 cell. (A) Family of whole-cell currents evoked under control conditions by 100-ms step depolarization from a holding potential of −70 mV. The step potentials are indicated on the left in the order of the observed currents. (B) Currents observed in the same cell 45 s after the start of superfusion with quercetin (20 μg/ml). (C) Plot of the current–voltage (I–V) relationship of the transient peak current observed at the beginning of the depolarization step under control conditions (open circles) and in the presence of quercetin (filled circles). (D) I–V plot of the late current component (95 ms after starting depolarization). (E) Plot of the time course of the quercetin effect. The peak transient inward Ca^{2+} current (T current) observed upon step depolarization from −70 to 0 mV is plotted before, during (bar) and after application of 20 μg/ml quercetin.

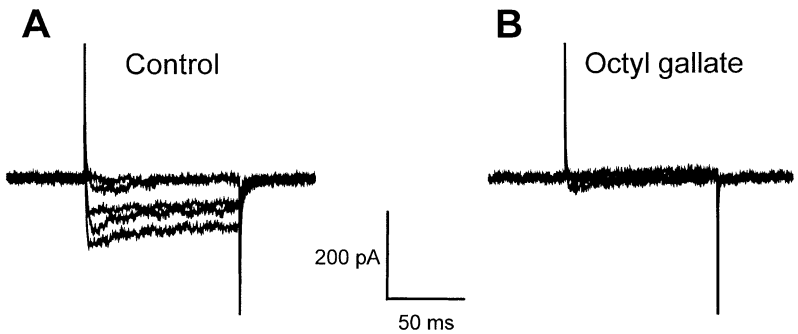

Fig. 5. Effect of octyl gallate on whole-cell currents in a CH_4C_1 cell. (A) The inward currents induced by step depolarization under control conditions were almost completely inhibited in the presence of octyl gallate (20 μg/ml) (B).

shown). The effect of octyl gallate was not reversible within the time span of the experiments (up to 10 min).

4. Discussion

This is the first time simple aromatic compounds and flavonoids from different subgroups have been systematically screened in order to compare the relative potencies of the compounds on VOCCs, using cultivated rat pituitary GH_4C_1 cells as a model. The rationale for using GH_4C_1 cells is that the calcium channels in these cells have been thoroughly characterized and the cells have been used in a multitude of studies on calcium channels (e.g., Xi et al., 1992; Fu et al., 1997; Peri et al., 2000), although in the future it would be interesting to use also arterial cell lines. The results of the present study indicate that several simple phenolic compounds and flavonoids are potent inhibitors of high K^+-evoked $^{45}Ca^{2+}$ entry, showing effects comparable to those of the clinically used compound verapamil. In addition, they showed an inhibitory activity against Ca^{2+} entry similar to that of other classes of natural Ca^{2+} channel antagonists, e.g. furanocoumarins (Vuorela, 1988). Another, rather astonishing, observation was that some flavonoids were surprisingly potent activators of Ca^{2+} entry. Our finding thus clearly shows that structurally related flavonoids do not necessarily exert similar actions on target cells.

In the light of our observations, it is of interest to consider the possible mechanisms of action of these compounds. Our voltage-clamp experiments showed that quercetin markedly enhanced both the transient and the delayed Ca^{2+} currents, indicating that quercetin may affect both the L- and T-type VOCCs. Quercetin also inhibits the contractile responses induced by increased extracellular Ca^{2+} in K^+-depolarized aortae and suppresses the spontaneous myogenic contractions recorded in portal veins (Duarte et al., 1993b). Other studies have shown that quercetin increases cellular cAMP (Lanza et al., 1987; Duarte et al., 1993b), and our results support these findings. Furthermore, as the protein kinase A inhibitor H-89 abolished the effect of quercetin on Ca^{2+} entry, our results suggest that the effect of quercetin on Ca^{2+} entry in GH_4C_1 cells is the result of an increased cAMP level in the cells followed by a protein kinase A-mediated activation of the VOCCs. Several studies have shown that phosphorylation of VOCCs by protein kinase A enhances Ca^{2+} entry. In addition, stimulation of GH_4C_1 cells with dibutyryl cAMP, or with forskolin, enhances Ca^{2+} entry through VOCCs (Törnquist, unpublished results).

Quercetin has been shown to relax smooth muscle in different test models, such as the main pulmonary artery contracted with adrenaline (Abdalla et al., 1989) and the central ear artery (Laekeman et al., 1986). Morales and Lozoya (1994) have shown that there are significant differences in the relaxant effect of quercetin in guinea pig and rat isolated aorta, the latter being more sensitive to quercetin. However, at low concentrations of quercetin they observed occasionally a slight contraction of aortic preparations. Our findings with GH_4C_1 cells are consistent with this as the contraction may be a consequence of the inhibitory activity of quercetin on Ca^{2+}-ATPase located in the endoplasmic reticulum (Fewtrell and Gomperts, 1977), and Ca^{2+}-ATPase in the plasma membrane (Gietzen et al., 1981) as well as the plasma membrane bound $[Na^+,K^+]$-ATPase (Kuriki and Racker, 1976). These effects should transiently decrease Ca^{2+} extrusion from the cytoplasm, leading to the observed contraction.

In rat uterine smooth muscle, the relaxing effect of quercetin on depolarization-evoked contractions was due to the quercetin-evoked increase of cAMP (Revuelta et al., 1997). Interestingly, quercetin has been reported to inhibit partly purified protein kinase A from rat brain (End et al., 1987). Thus, it is quite possible that the effect of quercetin may differ in different cell types.

Another mechanism of action of flavonoids is the inhibition of tyrosine-specific kinases (Akiyama et al., 1987; Smirnov and Aaronson, 1995). In GH_3 cells, genistein potently inhibited the high K^+-evoked Ca^2 current through VOCCs (Cataldi et al., 1996). It is conceivable that genistein also inhibited Ca^{2+} entry in our experiments by inhibiting tyrosine kinases. However, this may not be the

only mechanism of action, because daidzein (which does not inhibit tyrosine kinases; Cataldi et al., 1996) also evoked a substantial inhibition of Ca^{2+} entry in our experiments. It is interesting to note that quercetin and naringenin, which showed opposite effects on Ca^{2+} entry, both potently inhibited tyrosine kinase activity measured according to the method of Swarup et al. (1983) (Kéri, personal communication). Recently, genistein has also been shown to act as a vascular protective agent, i.e. genistein protected endothelial cells from the cytoxic effect of oxidized LDL (low-density lipoprotein) (Kapiotis et al., 1997).

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