ELSEVIER

European Journal of Pharmacology 329 (1997) 189–199

ejp

Differential effects of forskolin and 1,9-dideoxy-forskolin on nicotinic receptor- and K^+-induced responses in chromaffin cells

Luis Gandía [b], Maria Leiza Vitale [a], Mercedes Villarroya [b], Carmen Ramirez-Lavergne [a], Antonio G. García [b], José-María Trifaró [a,*]

[a] *Secretory Process Research Program, Department of Pharmacology, Faculty of Medicine, University of Ottawa, Ottawa, Canada K1H 8M5*
[b] *Departamento de Farmacología, Facultad de Medicina, and Servicio de Farmacología Clínica, Hospital de la Princesa, Universidad Autónoma de Madrid, C / Arzobispo Morcillo 4, 28029 Madrid, Spain*

Received 2 April 1997; accepted 8 April 1997

Abstract

The diterpene forskolin inhibits nicotine-evoked chromaffin cell Ca^{2+} influx, scinderin redistribution, F-actin disassembly and catecholamine secretion in a concentration-dependent (10–50 μM) fashion. On the other hand, forskolin showed weak inhibitory effects when the same responses were elicited by K^+-induced depolarization. Similar concentrations of 1,9-dideoxy-forskolin, a forskolin analog which does not activate adenylate cyclase, blocked very effectively the responses evoked by either of the two stimuli. Patch-clamp (whole-cell configuration) studies demonstrated that both diterpenes blocked fast and reversibly peak and total chromaffin cell nicotinic acetylcholine receptor currents, effects not mediated through adenylate cyclase activation. Moreover, both forskolin and 1,9-dideoxy-forskolin exhibited Ca^{2+} channel blocking properties. However, 1,9-dideoxy-forskolin was more potent than forskolin as a Ca^{2+} channel blocker. Furthermore, 1,9-dideoxy-forskolin was also more potent than forskolin as a nicotinic acetylcholine receptor and Ca^{2+} channel blocker and it was more potent as a nicotinic acetylcholine receptor blocker than Ca^{2+} channel blocker. The results showed powerful cAMP-independent effects of the diterpenes and suggest caution in interpretation of cAMP effects on chromaffin cells when its cellular levels are modified by forskolin. © 1997 Elsevier Science B.V.

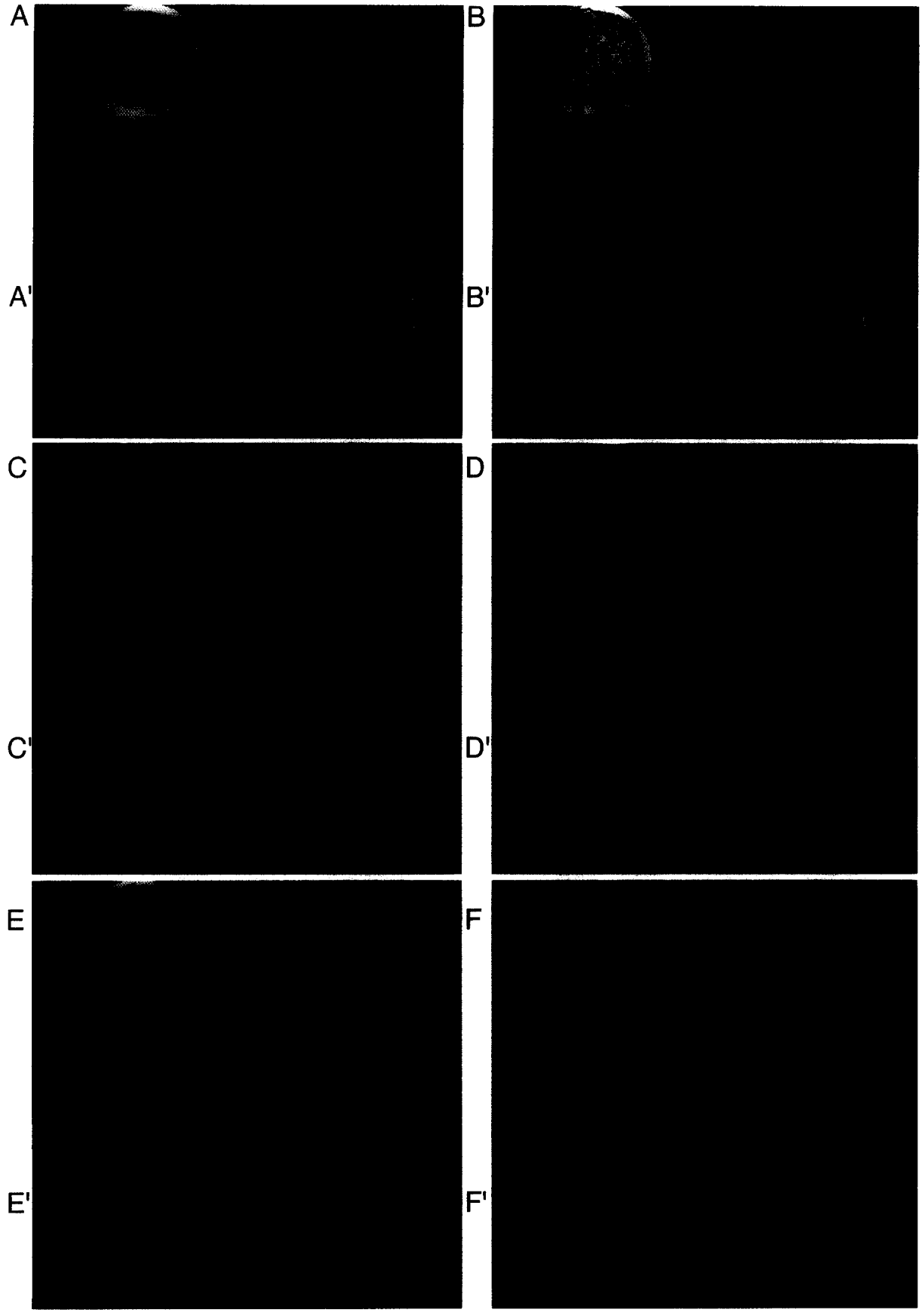
Keywords: Nicotinic receptor; Forskolin; 1,9-Dideoxy-forskolin; Chromaffin cell; Ca^{2+} channel

1. Introduction

Many contradictory reports on the role of cAMP on adrenal medullary chromaffin cells have been published (Jaanus and Rubin, 1974; Baker et al., 1985; Cheek and Burgoyne, 1987; Morita et al., 1987a,b; Chern et al., 1988; Higgins and Berg, 1988; Marriot et al., 1988; Wilson, 1988; Keogh and Marley, 1991; Marley et al., 1991; Anderson et al., 1992). Some of these contradictory findings might be partially due to the limited selectivity of some compounds used to enhance or decrease the intracellular levels of cAMP. A typical example is forskolin, a diterpene activator of adenylate cyclase (Seamon and Daly, 1986), widely used to increase the cytosolic levels of cAMP and to associate such an increase to a given functional cell response (Baker et al., 1985; Cheek and Burgoyne, 1987; Morita et al., 1987a,b; Chern et al., 1988; Marriot et al., 1988). Recently we initiated a systematic study to determine the role of cAMP in chromaffin cell scinderin redistribution, filamentous actin (F-actin) disassembly and catecholamine release in response to either nicotine stimulation or high K^+ depolarization. Chromaffin cells possess underneath the plasma membrane a mesh of F-actin (Lee and Trifaró, 1981; Trifaró et al., 1984; Cheek and Burgoyne, 1986) which is disassembled following nicotinic receptor stimulation or high K^+ depolarization (Cheek and Burgoyne, 1986; Trifaró et al., 1989; Vitale et al., 1991). Cortical F-actin disassembly during cell stimulation is the result of the activation of scinderin, a Ca^{2+}-dependent actin filament-severing protein, first discovered in chromaffin cells (Rodríguez Del Castillo et al., 1990) and expressed only in secretory tissues (Rodríguez Del Castillo et al., 1990; Tchakarov et al., 1990). Immunocytochemical experiments have shown that nicotinic receptor stimulation or chromaffin cell depolarization induces, simultaneously, cortical F-actin disassembly and redistribu-

* Corresponding author. Tel.: (1-613) 562-5421; Fax: (1-613) 562-5456.

PII S0014-2999(97)00111-8


A
B
A'
B'
C
D
C'
D'
E
F
E'
F'

tion of subplasmalemmal scinderin (Vitale et al., 1991; Rodríguez Del Castillo et al., 1992). These nicotine or high K^+-induced effects are Ca^{2+}-dependent and precede catecholamine release (Vitale et al., 1991).

The present results demonstrate that forskolin potently inhibited nicotine-evoked responses while K^+-induced responses were less affected. Though this could be due to a selective effect of the adenylate cyclase system on the nicotinic acetylcholine receptor, the possibility also existed that forskolin was exerting some direct pharmacological blocking effect on the nicotinic acetylcholine receptor. The experiments reported here prove that the last possibility was true. In addition, the results demonstrated that forskolin inhibits the whole-cell current through the nicotinic acetylcholine receptor, affecting to a much lesser degree the current through Ca^{2+} channels and that 1,9-dideoxy-forskolin, an analog unable to activate adenylate cyclase, blocked both currents effectively. These findings open new avenues for the chemical synthesis of novel compounds with nicotinic acetylcholine receptor and Ca^{2+} channel blocking properties.

2. Materials and methods

2.1. Chromaffin cell culture

Bovine adrenal glands were obtained from a local slaughterhouse and chromaffin cells were isolated by collagenase digestion and purified further using a Percoll gradient (Trifaró and Lee, 1980; Moro et al., 1990). Cells were plated at appropriate densities according to the specific study to be performed (plastic dishes, collagen-coated plastic dishes, collagen-coated glass coverslips) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5–10% fetal calf serum, 10 μM cytosine arabinoside and 10 μM fluorodeoxyuridine to prevent fibroblast proliferation. Cells were grown at 37°C in a humidified incubator under 5% CO_2 + air (95%) atmosphere. Cells were used 2–5 days after plating. Medium was first replaced 24 h after plating and then every 2–3 days.

2.2. Catecholamine release

Catecholamine release was measured by determining [^{3}H]noradrenaline output as described previously (Kenigsberg and Trifaró, 1980; Trifaró and Lee, 1980). Briefly chromaffin cells were plated on collagen-coated plastic 24-multiwell dishes at a density of 5×10^5 cells/well. Two- or three-day-old chromaffin cells were used. The cells were washed three times with special medium (in millimolar: NaCl, 110; KCl, 5; $MgSO_4$, 1; NaH_2PO_4, 1; Na-pyruvate, 1; $CaCl_2$, 2; $Fe(NO_3)_3$, 2.5×10^{-4}; ascorbic acid, 0.1; pH 7.2 (adjusted with HEPES) and 10% fetal calf serum) and incubated at room temperature for 5 min with 10^{-7} M [^{3}H]noradrenaline (from Amersham Canada; specific activity = 40 Ci/mmol). Cells were next washed by incubating the wells five times, 10 min each, with Locke's solution (in millimolar: NaCl, 154; KCl, 2.6; K_2HPO_4, 1.25; KH_2PO_4, 0.5; $MgCl_2$, 1.2; $CaCl_2$, 2; glucose, 10; pH 7.20). Cells were preincubated for 4 min with Locke's solution alone (control) or containing 20 μM forskolin or 1,9-dideoxy-forskolin. Immediately after the preincubation period cells were challenged for 40 s with Locke's solution containing 10^{-5} M nicotine or 56 mM K^+ in the presence or absence of either forskolin or 1,9-dideoxy-forskolin. To estimate basal release cells were incubated for 40 s with Locke's solution alone or containing forskolin or 1,9-dideoxy-forskolin. At the end of each incubation time, media were collected. [^{3}H]Noradrenaline cell content was determined by treating the cells with 10% trichloroacetic acid for 10 min followed by two washes of 6% trichloroacetic acid. Total [^{3}H]noradrenaline cell content was calculated by adding the [^{3}H]noradrenaline released during cell stimulation to the [^{3}H]noradrenaline extracted with trichloroacetic acid. As previously demonstrated, the detection limit of this assay is a concentration of [^{3}H]noradrenaline equivalent to 0.35% of the total cell catecholamine content (Vitale et al., 1991).

2.3. Fluorescence microscopy

Chromaffin cells were plated on collagen-coated coverslips contained within plastic Petri dishes at a density of

Fig. 1. Effects of nicotine stimulation and forskolin on scinderin (Sc) and F-actin (Ac) distribution in chromaffin cells: video-enhanced fluorescence and three-dimensional image analysis. Chromaffin cells cultured for 2 days were incubated for 4 min with Locke's solution (control) alone or containing either 20 μM forskolin or 20 μM 1,9-dideoxy-forskolin. Following this preincubation period, cells were challenged for 40 s with either 10^{-5} M nicotine or 56 mM K^+ in the absence or presence of each diterpene and immediately processed for fluorescence microscopy with scinderin antiserum No. 6 and rhodamine phalloidin. Video-enhanced image of cells incubated with Locke's solution alone (control) or containing either forskolin or 1,9-dideoxy-forskolin showed the characteristic cortical rings of fluorescence for F-actin (A) and scinderin (B). Three-dimensional image analysis of these cells showed uniform cortical fluorescent patterns for F-actin (A,A') and scinderin (B,B'). In the presence of nicotine, disrupted fluorescent rings were observed for F-actin (C) and scinderin (D). The cortical fluorescence intensity patterns for F-actin (C') and scinderin (D') show irregularities such as valleys and peaks. The valleys correspond to areas of F-actin disassembly or scinderin redistribution. The peaks, which have the same fluorescent intensity as controls, correspond to areas of intact cortical F-actin networks and normal scinderin distribution. Depolarization with high K^+ produced fluorescence patterns similar to those observed with nicotine stimulation (data not shown). Preincubation with 20 μM forskolin inhibited the changes evoked by nicotine (E,E', F,F'). Similarly, 1,9-dideoxy-forskolin inhibited the changes induced by both nicotine stimulation and K^+-evoked depolarization (data not shown).

3×10^5 cells/35 mm dish. Cultured cells were rinsed with Locke's solution. Cells were preincubated for 4 min with Locke's solution alone (control) or containing 20 μM forskolin or 1,9-dideoxy-forskolin. Immediately after the preincubation period cells were challenged 40 s with Locke's solution containing 10^{-5} M nicotine or 56 mM K^+ in the presence or absence of either forskolin or 1,9-dideoxy-forskolin. Preparations were immediately fixed in 3.7% formaldehyde for 20 min and processed for fluorescence microscopy (Lee and Trifaró, 1981). Briefly, cells were permeabilized by three successive exposures of 5 min each to 50, 100 and 50% acetone in water. Coverslips were next washed with phosphate-buffered saline (PBS, in millimolar: NaCl, 130; Na-phosphate 100; pH 7.0) and incubated for 60 min at room temperature with a blocking solution consisting of 1% bovine serum albumin in PBS. Chromaffin cells were double-stained for scinderin and F-actin. Cells were incubated with a polyclonal scinderin antibody (No. 6; 1:150 dilution in 0.1% bovine serum albumin in PBS) for 60 min at 37°C. The antibody was raised in rabbits against bovine scinderin and has been previously characterized (Rodríguez Del Castillo et al., 1990; Tchakarov et al., 1990; Vitale et al., 1991). Coverslips were thoroughly washed with PBS and further incubated with goat antirabbit IgG-fluorescein isothiocyanate conjugate (FITC-IgG, 1:250 dilution in 0.1% BSA in PBS; Sigma, St. Louis, MO, USA) for 60 min at 37°C. Preparations were washed with PBS and incubated 40 min at room temperature in the dark with rhodamine-phalloidin (0.25 U/ml dilution; Molecular Probes, Eugene, OR, USA). Finally, coverslips were rinsed with PBS and mounted in glycerol-PBS (1:1). Slides were observed with a Leitz Ortholux fluorescence microscope equipped with a Ploemopack II incident light illuminator, a L-filter block (KP 490 plus 1 μM GG 455 exciting filter, TK dichroic beam splitting mirror, K 515 suppression filter) for fluorescein and a M-filter block (2 μM BG plus S 546 exciting filter, TK 580 dichroic beam splitting mirror, K 580 suppression filter) for rhodamine. The cortical fluorescent rings (fluorescein = scinderin; rhodamine = F-actin) of 100 cells per coverslip were examined and classified as being 'continuous' or 'discontinuous'. Usually more than 6 coverslips (600 cells) per experimental condition were examined. The percentages of chromaffin cells showing cortical F-actin disassembly ('discontinuous' rhodamine ring) were calculated for each experimental condition. In order to avoid personal bias, code numbers were given to each coverslip. The cells were examined and classified without knowing whether they were from control or treated preparations. Only after all coverslips were examined, were the codes revealed to identify the experimental conditions used (single-blind design).

2.4. Video-enhanced image processing

Quantitative analysis of cortical rhodamine fluorescence (F-actin) was performed by using a Hamamatsu Photonic KK Argus-50/CL Image Processor (Hamamatsu Photonic Systems, Bridgewater, NJ, USA). The fluorescence microscope was coupled to the video camera (Carl Zeiss, TV3M model), which was connected to the Argus 50-Image processor. Video camera control parameters (i.e., gain, offset and sensitivity) were adjusted by using the image of a resting chromaffin cell on the monitor. Control parameters were set up to obtain a clear image of the cell on the

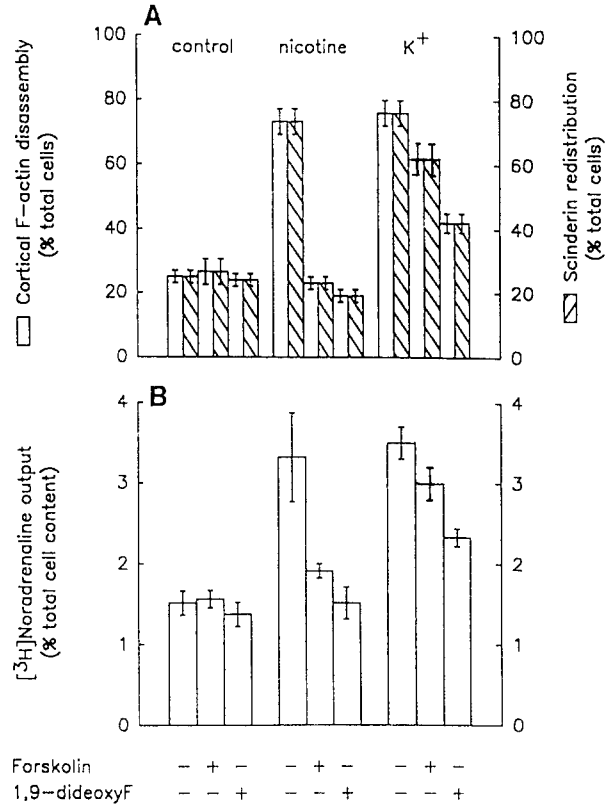


Fig. 2. Effects of forskolin and 1,9-dideoxy-forskolin on nicotine-evoked or high K^+-induced chromaffin cell scinderin redistribution, F-actin disassembly and catecholamine release. (A) Chromaffin cells grown for 2 days on collagen-coated coverslips were incubated for 4 min in the Locke's solution alone (control) or with Locke's solution containing either 20 μM forskolin or 20 μM 1,9-dideoxy-forskolin. Two groups of cells were stimulated also for an additional 40 s period with either 10^{-5} M nicotine or 56 mM K^+. Following the incubations, cells were fixed, permeabilized and processed for double-staining fluorescence microscopy using rhodamine-labelled phalloidin (a probe for F-actin) and scinderin antiserum No. 6 as indicated in Section 2. One hundred cells per coverslip were examined for scinderin (fluorescein staining) or for F-actin (rhodamine fluorescence) peripheral distribution and were classified as having a 'continuous' (resting) or 'patched' cortical staining (scinderin redistribution and F-actin disassembly) as indicated in Section 2. Each bar represents the mean ± S.E.M. of the percentage of discontinuous scinderin and F-actin distribution of 5–8 coverslips (500–800 cells for each value) containing cells from 3 different cultures. (B) Chromaffin cells also grown for 2 days on collagen-coated plastic multiwell dishes were labelled with [^{3}H]noradrenaline as indicated in Section 2. Cells were incubated with either 20 μM forskolin or 20 μM 1,9-dideoxy-forskolin and stimulated following the same protocol described in (A). [^{3}H]Noradrenaline output was measured as described in Section 2. Each bar represents the mean ± S.E.M. of values obtained from 5–7 different culture wells.

monitor and fluorescence intensity of 250 (arbitrary units) in the cortical region of the cell. Image analysis of other cells was carried out without modifying the video camera control parameters. The three-dimensional graphic analysis represents the coordinates of the equatorial plane of the cell as the X and Y axes and the fluorescence intensity of this plane as the Z axis (Vitale et al., 1995). The image stored in the memory is constructed of 16 bits containing 512 horizontal pixels by 483 vertical pixels.

2.5. Measurements of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake studies were carried out in cells after 2–3 days in culture. Before the experiment, cells were washed twice with 0.5 ml Krebs-HEPES solution (in millimolar): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 11, HEPES 10, pH 7.2, at 37°C. $^{45}Ca^{2+}$ uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}CaCl_2$ at a final concentration of 3 μCi ml^{-1} in the presence of Krebs-HEPES (basal uptake), high K^+ solution (Krebs-HEPES containing 70 mM KCl with isosmotic reduction of NaCl), or 100 μM dimethyl-phenyl-piperazinium (DMPP) in Krebs-HEPES. Before stimulation, cells were preincubated with either 20 μM forskolin or 20 μM 1,9-dideoxy-forskolin for 10 min. In some cases, $^{45}Ca^{2+}$ uptake induced by high K^+ was tested in the presence of the L-type Ca^{2+} channel blocker, furnidipine. This incubation was carried out during 1 min and at the end of this period the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.5 ml of a cold Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals. To measure the radioactivity retained by chromaffin cells, cells were scraped off the plates with a plastic pipette tip after adding 0.5 ml 10% trichloroacetic acid and 3.5 ml of scintillation

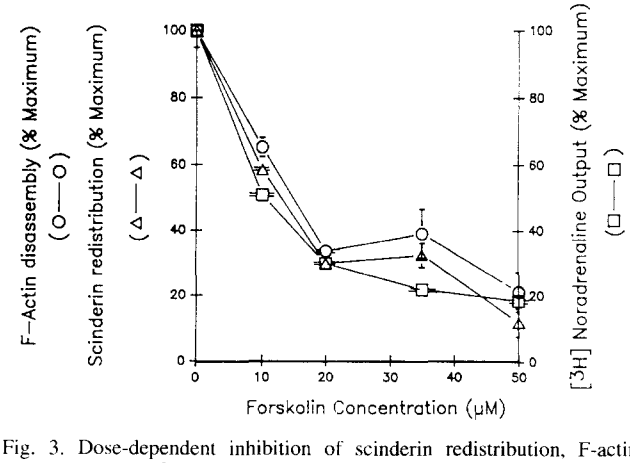


Fig. 3. Dose-dependent inhibition of scinderin redistribution, F-actin disassembly and [^{3}H]noradrenaline output in nicotine-stimulated cells in the presence of increasing concentrations of forskolin (10, 20, 35, or 50 μM) for 40 s. Each data point depicted represents the mean ± S.E.M. of results obtained from 300–700 cells for fluorescence microscopy studies and the mean ± S.E.M. results obtained in 4–8 culture dishes from 2 different cell cultures for [^{3}H]noradrenaline output studies.

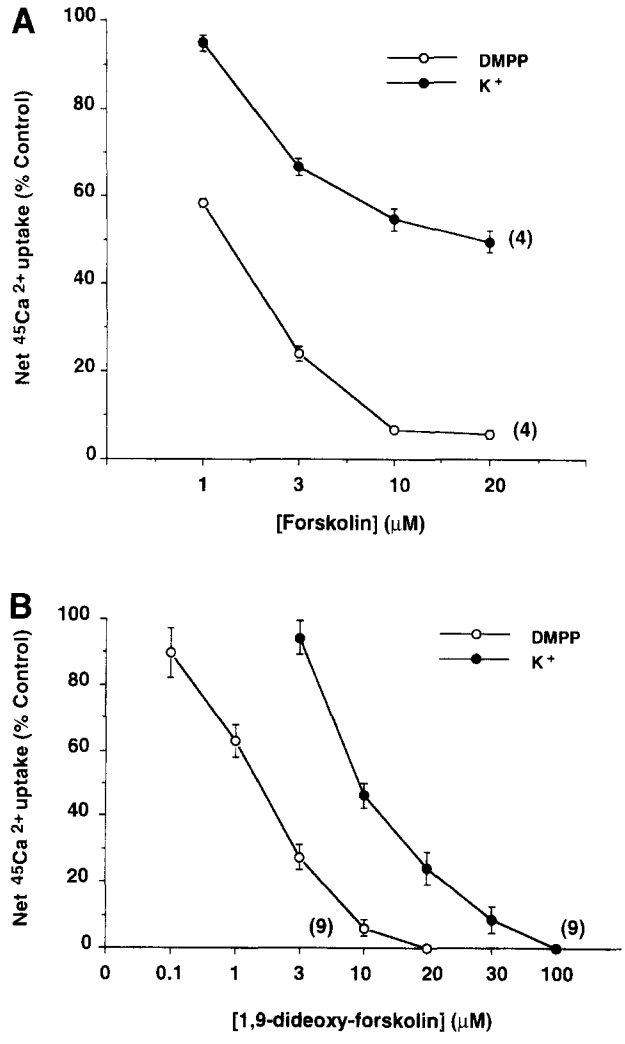


Fig. 4. Effects of forskolin (panel A) or 1,9-dideoxy-forskolin (panel B) on $^{45}Ca^{2+}$ uptake into cultured chromaffin cells stimulated with 70 mM K^+ or 100 μM dimethyl-phenyl-piperazinium (DMPP) for 60 s. Before stimulation, cells were preincubated with each concentration of drug for 10 min. In each individual experiment, $^{45}Ca^{2+}$ uptake (ordinate scale) was normalized to 100% ($^{45}Ca^{2+}$ taken up by cells in the absence of drug). Data are means ± S.E.M. of the number of wells shown in parentheses from 3 different cell batches.

fluid (Ready Micro, Beckman) was added. The radioactivity of the samples was measured in a Packard beta counter. Results are expressed as % of net evoked Ca^{2+} taken up by control cells.

2.6. Measurements of whole-cell currents

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (22–24°C) with control Tyrode solution containing (in millimolar): NaCl 137, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, pH 7.4 titrated

with NaOH. For whole-cell recording of dimethyl-phenyl-piperazinium-induced currents (I_{DMPP}), brief (250 ms) pulses with an extracellular solution containing 100 μM dimethyl-phenyl-piperazinium were applied to a chromaffin cell voltage-clamped at −80 mV by means of a fast superfusion system (described below). For whole-cell recordings of Ba^{2+} currents through Ca^{2+} channels, 10 mM Ba^{2+} was used as the charge carrier and 5 μM tetrodotoxin (TTX) was added to the perfusion solution. Cells were dialyzed with a standard intracellular solution containing (in millimolar): NaCl 10, CsCl 110, TEA-Cl 20, EGTA 0–14, HEPES 20, Mg · ATP 5, GTP 0.3, pH 7.2 titrated with CsOH.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 patch-clamp amplified, allowing cancellation of capacitative transients and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pClamp software (Axon Instruments, Forster City, CA, USA) were used to acquire and analyze the data. Control and test solutions were exchanged using a multi-barrelled concen-

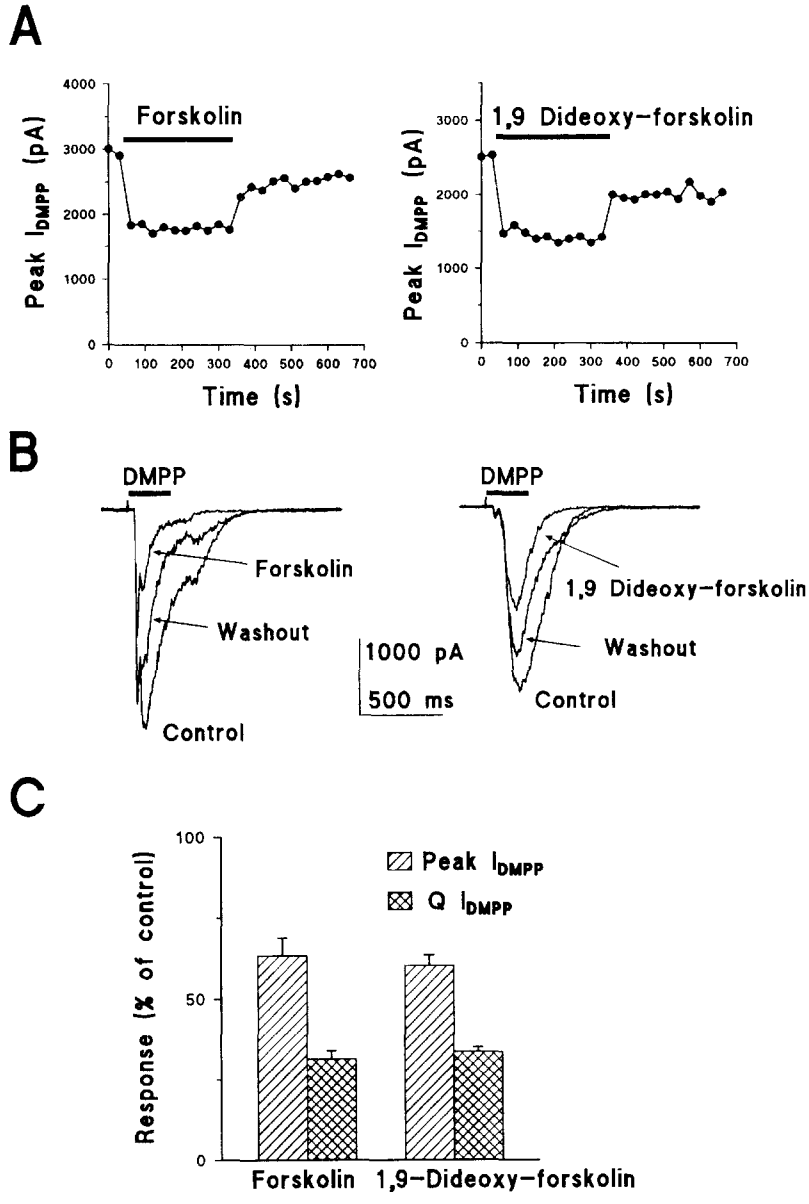


Fig. 5. Effects of forskolin and its analogue 1,9-dideoxy-forskolin on dimethyl-phenyl-piperazinium (DMPP)-induced whole-cell currents in cultured chromaffin cells. Panel A shows the time-course of the blocking effects of 20 μM forskolin (left) and 20 μM 1,9-dideoxy-forskolin on the peak I_{DMPP} recorded in two different chromaffin cells. In both cases, the blocking effects had a fast onset of action and a fast but partial recovery upon washout of the drug from the extracellular solution. Panel B shows original current records obtained before, during drug application or after washout of the drug from the perfusion solution. Panel C shows average results obtained when measuring the blocking effects of both drugs on peak I_{DMPP} or when measuring the total charge (Q) entering the cell. Data are means ± S.E.M. of 10 cells for each drug.

tration-clamp device, the common outlet was placed within 100 μm of the cell to be patched. The flow rate (0.2–0.5 ml^{-1}) was regulated by gravity to achieve a complete replacement of the cell surroundings within less than 1 s. Cells were clamped at −80 mV holding potential. Step depolarizations to various test potentials from this holding potential lasted 25–50 ms and were applied at 20 s intervals to minimize the rundown of Ca^{2+} currents (Fenwick et al., 1982). Cells with pronounced rundown were discarded.

2.7. cAMP assay

cAMP levels were measured in acid-ethanol extracts of chromaffin cells following the procedure described by Brown et al. (1971).

2.8. Materials and solutions

The following materials were used: collagenase from *Clostridium histolyticum* (Boehringer-Mannheim); bovine serum albumin fraction V; cytosine arabinoside, fluoro-deoxyuridine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and EGTA (Sigma); fetal calf serum penicillin and streptomycin (Gibco); scintillation fluid Ready micro (Beckman; $^{45}Ca^{2+}$ (specific activity 10–40 mCi mg^{-1} calcium, Amersham). Forskolin and 1,9-dideoxy-forskolin were from Research Biochemical International (RBI). All other chemicals were reagent grade. Forskolin and 1,9-dideoxy-forskolin were dissolved in dimethyl sulfoxide (Merck) at 10^{-1} M and diluted in saline solutions to the desired concentrations. The highest concentration of dimethyl sulfoxide used (not more than 0.1%) had no effects on all parameters studied.

3. Results

3.1. Effects of forskolin and 1,9-dideoxy-forskolin on nicotine- and high K^+-evoked chromaffin cell scinderin redistribution, F-actin disassembly and catecholamine release

Immunocytochemical and video-enhanced microscopy studies in chromaffin cells cultured for 2 days and stained

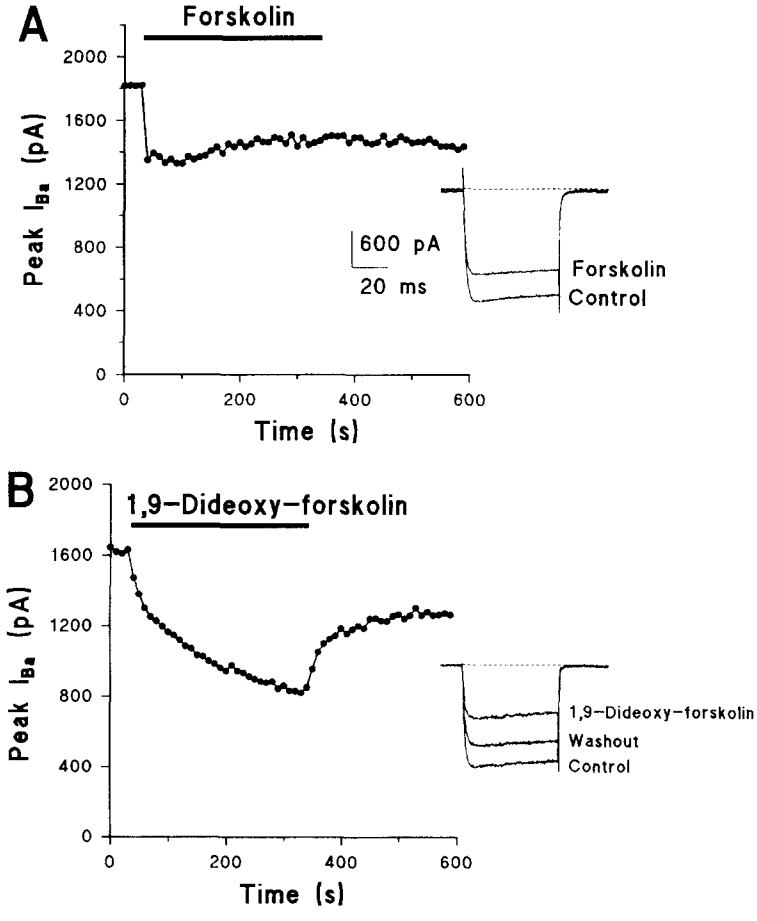


Fig. 6. Effects of forskolin and 1,9-dideoxy-forskolin on I_{Ba} recorded in two different cultured chromaffin cells. Cells were voltage-clamped at −80 mV holding potential and I_{Ba} were elicited by depolarizing pulses to +10 mV applied during 50 ms at 10 s intervals. Panel A shows the time-course of the blocking effects of forskolin (20 μM) applied during the time indicated by the horizontal bar. Inset shows capacitative and leak-subtracted current traces obtained before, during and after washout of forskolin. Panel B shows the effects of superfusion of a bovine chromaffin cell with a solution containing 20 μM 1,9-dideoxy-forskolin during the time indicated by the horizontal bar. Inset shows capacitative and linear leak-subtracted current traces obtained under the conditions indicated in the figure.

with scinderin antibodies, followed by FITC-IgG and rhodamine-labelled phalloidin, permit both scinderin and filamentous actin (F-actin) to be visualized. Measurements of fluorescence intensity by video-enhanced microscopy demonstrates that, under resting conditions, both F-actin and scinderin display a high level of continuous fluorescence in the cortical region of the cells (Fig. 1A, A′ and B, B′) in agreement with previous findings (Vitale et al., 1991). Upon stimulation for 40 s with either 10 μM nicotine or a depolarizing concentration of K^+ (56 mM), F-actin and scinderin cortical fluorescent rings become fragmented and the cell exhibits a 'patchy' F-actin and scinderin fluorescence (Fig. 1C, D). The decrease in fluorescence indicates cortical scinderin redistribution and cortical F-actin disassembly and they appear in image analysis as valleys of low fluorescence intensity (Fig. 1C′, D′). The peaks correspond to the fluorescent patches observed in cells shown in Fig. 1C and D. The intensity of the fluorescent peaks was 250 arbitrary units. This value is similar to the intensity of the cortical fluorescent pattern observed in control cells (Fig. 1A′, B′). The percentage of cells displaying scinderin redistribution and cortical F-actin disassembly upon 40 s stimulation with either 10 μM nicotine or 56 mM K^+ is shown in Fig. 2A. It can be observed that the values for scinderin redistribution and F-actin disassembly are identical since all cells showing scinderin redistribution also display F-actin disassembly. The two responses to chromaffin cell stimulation are also accompanied by an increase in [^{3}H]noradrenaline output, as shown in Fig. 2B, for cells stimulated for 40 s with either nicotine or high K^+. Forskolin (20 μM) alone did not affect scinderin re-distribution, cortical F-actin disassembly and basal catecholamine secretion (Fig. 2A, B). Image analysis showed 3-dimensional patterns similar to those obtained with control cells (Fig. 1A′, B′). To evaluate the possible modulatory role of cAMP in nicotine or high K^+-mediated responses, forskolin, a diterpene known to stimulate adenylate cyclase (Seamon and Daly, 1986), was tested. cAMP levels in chromaffin cells increase in a dose-dependent manner upon incubation of cells for 40 s with increasing concentrations of forskolin (control = 1.9 ± 0.02, 2.2 ± 0.01, 2.55 ± 0.02, 2.85 ± 0.1 and 3.2 ± 0.03 pmol/10^6 cells ($n = 4$), for 10, 20, 35 and 50 μM forskolin). On the other hand, stimulation for 40 s with 10 μM nicotine did not modify chromaffin cell cAMP levels (1.8 ± 0.08; $n = 4$). Forskolin was next tested on the three nicotine-induced responses (scinderin redistribution, F-actin disassembly and catecholamine release). Fig. 3 shows a concentration-dependent inhibition by forskolin of the three nicotine-induced responses. The effects of forskolin on high K^+-induced responses were also tested. However, in this case, forskolin was not a very effective inhibitor of chromaffin cell scinderin redistribution (19% inhibition), F-actin disassembly (19% inhibition) and catecholamine release (16% inhibition) in response to depolarization with 56 mM K^+ (Fig. 2A, B). In view of these findings, 1,9-dideoxy-forskolin was tested for its effects on chromaffin cell responses to nicotine and high K^+ stimulation. 1,9-Dideoxy-forskolin is unable to stimulate adenylate cyclase and, consequently, it does not affect cAMP levels. 1,9-Dideoxy-forskolin alone did not affect scinderin redistribution, F-actin disassembly and basal catecholamine secretion (Fig. 2A, B). However, 1,9-dideoxy-forskolin was as effective as forskolin in blocking the three nicotine-evoked responses in chromaffin cells (Fig. 2A, B). Moreover, and contrary to the weak inhibitory effects of forskolin, 1,9-dideoxy-forskolin produced a very significant inhibition of high K^+-evoked chromaffin cell scinderin redistribution, F-actin disassembly and catecholamine release (Fig. 2A, B). The different effects of forskolin and 1,9-dideoxy-forskolin on high K^+-evoked chromaffin responses were then further investigated (see below).

3.2. Effects of forskolin and 1,9-dideoxy-forskolin on $^{45}Ca^{2+}$ uptake into cultured chromaffin cells

Stimulation conditions which produced a maximum $^{45}Ca^{2+}$ uptake above basal levels were selected (Gandía et al., 1991). Therefore, cells were stimulated for 60 s with 100 μM dimethyl-phenyl-piperazinium or 70 mM K^+ in the presence of $^{45}Ca^{2+}$. Under these conditions, the net increase above basal levels of $^{45}Ca^{2+}$ taken up was 3- to 5-fold. Forskolin (Fig. 4A) inhibited the $^{45}Ca^{2+}$ uptake induced by dimethyl-phenyl-piperazinium in a concentration-dependent manner with an IC_{50} of 3.9 μM. In contrast, forskolin was not a powerful inhibitor of K^+-evoked $^{45}Ca^{2+}$ uptake. At the concentration of 50 μM forskolin produced a 50% blockade of K^+-induced $^{45}Ca^{2+}$ uptake whereas 10 μM forskolin inhibited $^{45}Ca^{2+}$ uptake by 98% in response to dimethyl-phenyl-piperazinium. 1,9-Dideoxy-forskolin also inhibited dimethyl-phenyl-piperazinium-induced Ca^{2+} entry in a concentration-dependent manner (Fig. 4B), exhibiting an IC_{50} of 2.1 μM. Moreover, 1,9-dideoxy-forskolin inhibited the K^+-evoked $^{45}Ca^{2+}$ uptake in a concentration-dependent manner, showing an IC_{50} of 8.3 μM.

3.3. Effects of forskolin and 1,9-dideoxy-forskolin on I_{DMPP}

Fig. 5A shows the time-course of the blocking effects of forskolin (left panel) and of 1,9-dideoxy-forskolin (right panel) on peak I_{DMPP}. Dimethyl-phenyl-piperazinium currents were triggered by the application at 30 s intervals of 250 ms pulses of dimethyl-phenyl-piperazinium (100 μM) to a voltage-clamped chromaffin cell (holding potential −80 mV). With such brief applications of dimethyl-phenyl-piperazinium, reproducible current could be elicited for at least a 15–20 min period. Average amplitude of peak I_{DMPP} was 2112 ± 184 pA ($n = 16$ cells). Upon superfusion of forskolin, I_{DMPP} was rapidly and partially suppressed. In 8 cells, forskolin (20 μM) blocked I_{DMPP} by $37 \pm 5\%$. Blocking effects of forskolin were almost

fully reversible upon washout of the drug from the extracellular solution (Fig. 5A, left panel). Similar results were obtained when cells were superfused with the analogue 1,9-dideoxy-forskolin (20 μM). Again, a fast and partial blockade was observed (40 ± 3%; $n = 8$ cells), being such blockade partially reversible upon removal of the drug. Fig. 5B shows original traces of I_{DMPP} recorded before, during superfusion of the cells with both drugs and after washout of the drugs. As shown, blockade of peak current was also accompanied by a greater decrease in the area of the total current entering the cell. In control conditions, average charge amounted to 404 ± 38 pC ($n = 16$). Forskolin (20 μM) decreased total charge by 69 ± 2% while its analogue 1,9-dideoxy-forskolin induced a 66 ± 1% blockade (Fig. 5C).

3.4. Effects of forskolin and 1,9-dideoxy-forskolin on I_{Ba}

Direct blocking effects of forskolin and 1,9-dideoxy-forskolin on Ca^{2+} channels were also tested. Ba^{2+} currents through Ca^{2+} channels (I_{Ba}) were elicited by 50 ms depolarizing pulses to + 10 mV, given at 10 s intervals to a voltage-clamped chromaffin cell (−80 mV holding potential). Fig. 6A shows the time-course of I_{Ba} and the blocking effects induced by forskolin (20 μM). I_{Ba} stabilized at about 1800 pA and upon superfusion with forskolin, a 22% decrease was observed. In 8 cells, forskolin blocked by 22 ± 3% I_{Ba}, from a control value of 1211 ± 171 pA to 945 ± 160 pA. Inset shows current traces obtained before, during and after forskolin treatment. On the other hand, superfusion of the cells with 1,9-dideoxy-forskolin (20 μM) induced a progressive decrease of I_{Ba} that amounted to 41 ± 7% ($n = 8$ cells) after 5 min superfusion with the drug (Fig. 6B). Blockade was partially reversible upon washout of the drug from the extracellular solution. The inset shows current traces obtained in the three conditions indicated. As shown, 1,9-dideoxy-forskolin did not induce significant changes in the activation and inactivation kinetics of I_{Ba} on this time scale.

4. Discussion

The role of cAMP on catecholamine secretion from the chromaffin cell has been extensively studied (Jaanus and Rubin, 1974; Baker et al., 1985; Cheek and Burgoyne, 1987; Morita et al., 1987a,b; Chern et al., 1988; Higgins and Berg, 1988; Marriot et al., 1988; Wilson, 1988; Keogh and Marley, 1991; Marley et al., 1991; Anderson et al., 1992); however, conflicting results on the effects of the nucleotide on secretion have been obtained. Some of the published papers demonstrated a facilitatory role of cAMP in secretion (Morita et al., 1987a,b; Chern et al., 1988; Wilson, 1988) whereas other publications show either no effects (Jaanus and Rubin, 1974) or an inhibitory role for cAMP in chromaffin cell secretion (Baker et al., 1985; Cheek and Burgoyne, 1987). In several of these studies, forskolin was used to elevate chromaffin cell cAMP levels (Baker et al., 1985; Cheek and Burgoyne, 1987; Morita et al., 1987a,b; Chern et al., 1988; Marriot et al., 1988).

Forskolin was first introduced as a cardio-selective compound with reversible adenylate cyclase stimulating activities (Laurenza et al., 1989). Therefore, forskolin has been extensively used as a tool to increase cellular levels of cAMP (Laurenza et al., 1989). However, it became apparent in recent years that forskolin also produces cAMP-independent effects (Sergeant and Kim, 1985; Hoshi et al., 1988; White, 1988; Aylwin and White, 1992; Hartzell and Budnitz, 1992; Park and Kim, 1996). The effects of forskolin on chromaffin cells are not exceptions and although forskolin elevates cAMP levels in these cells, its effects on scinderin redistribution, F-actin disassembly and secretion are not mediated through adenylate cyclase activation as the results presented here clearly indicate. The first observation from these studies is that forskolin is able to block very effectively all nicotine-evoked responses (Ca^{2+} influx, scinderin redistribution, F-actin disassembly and secretion). Forskolin was a weak inhibitor when responses were elicited by high K^+. On the contrary, 1,9-dideoxy-forskolin at the same concentration (20 μM) was able to block effectively both nicotinic acetylcholine receptor-evoked and high K^+-induced responses. Because 1,9-dideoxy-forskolin does not activate the adenylate cyclase (Laurenza et al., 1989), the first conclusion drawn from these studies is that the inhibitory effects of both forskolin and 1,9-dideoxy-forskolin on nicotinic acetylcholine receptor-evoked chromaffin cell responses are not mediated by elevated cAMP levels as a result of adenylate cyclase activation. The second conclusion from these studies is that 1,9-dideoxy-forskolin was more effective than forskolin in blocking the responses to high K^+. The only structural difference between forskolin and 1,9-dideoxy-forskolin is the absence in the latter compound of OH groups at positions 1 and 9. This structural difference might be responsible for the differing effects observed between these two diterpenes.

Effects of forskolin on nicotinic acetylcholine receptor responses have been reported recently. Published evidence has shown that forskolin increases the rate of nicotinic acetylcholine receptor desensitization in rat soleus and frog sartorius muscles (Albuquerque et al., 1986; Middleton et al., 1986). It was concluded from these studies that an elevation of intracellular cAMP was responsible for receptor desensitization. On the other hand, it has been shown that micromolar concentrations of forskolin, acting probably as a local anaesthetic, altered the gating properties of the *Torpedo* nicotinic acetylcholine receptor expressed in *Xenopus* oocytes (White, 1988). Similarly, a direct anaesthetic-like effect of forskolin on nicotinic acetylcholine receptor-induced $^{86}Rb^+$ influx into PC_{12} cells has been demonstrated (McHugh and McGee, 1986). The patch-clamp studies presented here clearly demonstrate that both forskolin and 1,9-dideoxy-forskolin fast and reversibly

blocked nicotinic acetylcholine receptor peak and total currents. These results demonstrated a direct effect of the diterpenes on the chromaffin cell nicotinic acetylcholine receptors. The site of action of these compounds on chromaffin cell nicotinic acetylcholine receptors remains to be elucidated. However, published studies on chick myotubes suggest that forskolin interacts with the same receptor binding sites of [^{3}H]phencyclidine, a non-competitive antagonist (Häggblad et al., 1987). The same study also showed that forskolin failed to block α-bungarotoxin binding in chick myotubes.

Differential effects between forskolin and 1,9-dideoxy-forskolin are clearly illustrated by their effects on K^+-evoked responses. Forskolin was a weak inhibitor of $^{45}Ca^{2+}$ influx into chromaffin cells in response to high K^+ whereas 1,9-dideoxy-forskolin was a very effective blocker. Moreover, the fact that forskolin blocked quite well $^{45}Ca^{2+}$ influx in response to nicotinic acetylcholine receptor stimulation suggests that, as expected, Ca^{2+} influx through Ca^{2+} channels is secondary to nicotinic acetylcholine receptor activation.

The patch-clamp studies described here also show that 1,9-dideoxy-forskolin blocks whole-cell Ca^{2+} currents while the same concentration of forskolin was much less effective. The fact that 1,9-dideoxy-forskolin was a much better blocker of chromaffin cell Ca^{2+} currents would explain the different pharmacological behaviour between the two diterpenes. It should also be pointed out that 1,9-dideoxy-forskolin is a more powerful blocker of nicotinic acetylcholine receptors than Ca^{2+} channels, since as shown in Fig. 4, 1,9-dideoxy-forskolin was more potent in inhibiting Ca^{2+} influx in response to nicotinic acetylcholine receptor stimulation (IC_{50} 2.1 μM) than K^+-evoked depolarization (IC_{50} 8.3 μM). The experiments also showed that 1,9-dideoxy-forskolin is more potent than forskolin on its effects on nicotinic acetylcholine receptors since IC_{50} values for Ca^{2+} influx were 2.1 μM and 3.9 μM, respectively.

In conclusion, the experiments described here clearly indicate that, in the study of cAMP effects on chromaffin cells, the results should be interpreted with caution when forskolin is used to increase cAMP intracellular levels. The results show direct blocking effects on chromaffin cell nicotinic acetylcholine receptors by forskolin and 1,9-dideoxy-forskolin, an analog without adenylate cyclase activating properties. The data presented here also indicate an additional and powerful Ca^{2+} channel blocking effect of 1,9-dideoxy-forskolin, and that this effect is responsible for the different degree of blockade produced by the two diterpenes on nicotinic receptor- and high K^+-evoked responses.

Acknowledgements

We are grateful to Ms. Tang for technical assistance in the preparation of chromaffin cell cultures and to Ms. S.J. Dunn for typing the manuscript. This work was supported by grants from the Medical Research Council of Canada to J.-M.T. and from Fundación Ramón Areces, and DGJCTT No. PB94-0150 to A.G.G.

References

Albuquerque, E.X., Deshpande, S.S., Aracqva, Y., Alkondon, M., Daly, J.W., 1986. A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. FEBS Lett. 199, 113.

Anderson, K., Robinson, P.J., Marley, P.D., 1992. Cholinoceptor regulation of cyclic AMP levels in bovine adrenal medullary cells. Br. J. Pharmacol. 106, 360.

Aylwin, M.L., White, M.M., 1992. Forskolin acts as a noncompetitive inhibitor of nicotinic acetylcholine receptors. Mol. Pharmacol. 41, 906.

Baker, E.M., Cheek, T.R., Burgoyne, R.D., 1985. Cyclic AMP inhibits secretion from bovine adrenal chromaffin cells evoked by carbamylcholine but not by high K^+. Biochim. Biophys. Acta 846, 388.

Brown, B.L., Albano, J.D.M., Ekins, R.P., Sgherzi, A.M., 1971. A simple and sensitive saturation assay method for the measurement of adenosine 3′,5′ cyclic monophosphate. Biochem. J. 121, 561.

Cheek, T.R., Burgoyne, R.D., 1986. Nicotine-evoked disassembly of cortical actin filaments in adrenal chromaffin cells. FEBS Lett. 207, 110.

Cheek, T.R., Burgoyne, R.D., 1987. Cyclic AMP inhibits both nicotine-induced actin disassembly and catecholamine secretion from bovine adrenal chromaffin cells. J. Biol. Chem. 262, 11663.

Chern, Y.-J., Kim, K.T., Slakey, L.L., Westhead, E.W., 1988. Adenosine receptors activate adenylate cyclase and enhance secretion from bovine adrenal chromaffin cells in the presence of forskolin. J. Neurochem. 50, 1484.

Fenwick, E.M., Marty, A., Neher, E., 1982. Sodium and calcium channels in bovine chromaffin cells. J. Physiol. 331, 599.

Gandía, L., Casado, L.F., López, M.G., García, A.G., 1991. Separation of two pathways for calcium entry into chromaffin cells. Br. J. Pharmacol. 103, 1073.

Häggblad, J., Eriksson, H., Hedlund, B., Heilbronn, E., 1987. Forskolin blocks carbachol-mediated ion-permeability of chick myotube nicotinic receptors and inhibits binding of ^{3}H-phencyclidine to *Torpedo* microsac nicotinic receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 336, 381.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflüg. Arch. 391, 85.

Hartzell, H.C., Budnitz, D., 1992. Differences in effects of forskolin and an analog on calcium currents in cardiac myocytes suggest intra- and extracellular sites of action. Mol. Pharmacol. 41, 880.

Higgins, L.S., Berg, D.K., 1988. Cyclic AMP dependent mechanism regulates acetylcholine receptor function on bovine adrenal chromaffin cells and discriminates between new and old receptors. J. Cell Biol. 107, 1157.

Hoshi, T., Garber, S.S., Aldrich, R.W., 1988. Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation. Science 240, 1652.

Jaanus, S.D., Rubin, R.P., 1974. Analysis of the role of cyclic adenosine 3′-5′-monophosphate in catecholamine release. J. Physiol. (London) 237, 465.

Kenigsberg, R.L., Trifaró, J.-M., 1980. Presence of high affinity uptake for catecholamines in cultured bovine adrenal chromaffin cells. Neuroscience 5, 1547.

Keogh, R., Marley, P.D., 1991. Regulation of cyclic AMP levels by calcium in bovine adrenal medullary cells. J. Neurochem. 57, 1721.

Laurenza, A., McHugh-Sutkowski, E., Seamon, K.B., 1989. Forskolin: a specific stimulator of adenyl cyclase or a diterpene with multiple sites of action?. Trends Pharmacol. Sci. 10, 442.

Lee, R.W.H., Trifaró, J.-M., 1981. Characterization of anti-actin antibodies and their use in immunocytochemical studies on the localization of actin in adrenal chromaffin cells. Neuroscience 6, 2087.

Marley, P.D., Thomson, K.A., Jachno, K., Johnston, M.J., 1991. Histamine-induced increases in cyclic AMP levels in bovine adrenal medullary cells. Br. J. Pharmacol. 104, 839.

Marriott, D., Adams, M., Boarder, M.R., 1988. Effect of forskolin and prostaglandin E_1 on stimulus secretion coupling in cultured bovine adrenal chromaffin cells. J. Neurochem. 50, 616.

McHugh, E.M., McGee, R. Jr., 1986. Direct anesthetic-like effects of forskolin on the nicotinic acetylcholine receptor of PC_{12} cells. J. Biol. Chem. 261, 3103.

Middleton, P., Jaramillo, F., Schuetze, S.M., 1986. Forskolin increases the rate of acetylcholine receptor desensitization at rat soleus endplates. Proc. Natl. Acad. Sci. USA 83, 4967.

Morita, K., Doshi, T., Kitayama, S., Koyama, Y., Tsujimoto, A., 1987a. Enhancement of stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells by forskolin. J. Neurochem. 48, 243.

Morita, K., Doshi, T., Kitayama, S., Koyama, Y., Tsujimoto, A., 1987b. Stimulation-evoked Ca^{2+} fluxes in cultured bovine adrenal chromaffin cells are enhanced by forskolin. J. Neurochem. 48, 248.

Moro, M.A., López, M.G., Gandía, L., Michelena, P., García, A.G., 1990. Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. Anal. Biochem. 185, 243.

Park, T.-J., Kim, K.-T., 1996. Cyclic AMP-independent inhibition of voltage-sensitive calcium channels by forskolin in PC_{12} cells. J. Neurochem. 66, 83.

Rodríguez Del Castillo, A., Lemaire, S., Tchakarov, L., Jeyapragasan, M., Doucet, J.P., Vitale, M.L., Trifaró, J.-M., 1990. Chromaffin cell scinderin: a novel calcium-dependent actin-filament severing protein. EMBO J. 9, 43.

Rodríguez Del Castillo, A., Vitale, M.L., Trifaró, J.-M., 1992. Ca^{2+} and pH determine the interaction of chromaffin cell scinderin with phosphatidylserine and phosphatidylinositol 4,5,-bisphosphate and its cellular distribution during nicotinic-receptor stimulation and protein kinase C activation. J. Cell Biol. 119, 797.

Seamon, K.B., Daly, J.W., 1986. Forskolin: its biological and chemical properties. In: Greengard, P., Robinson, G.A. (Eds.), Advance in Cyclic Nucleotide and Protein Phosphorylation Research. Raven Press, New York, NY, p. 1.

Sergeant, S., Kim, H.D., 1985. Inhibition of 3-*O*-methylglucose transport in human erythrocytes by forskolin. J. Biol. Chem. 260, 14677.

Tchakarov, L., Vitale, M.L., Jeyapragasan, M., Rodríguez Del Castillo, A., Trifaró, J.-M., 1990. Expression of scinderin, an actin filament severing protein in different tissues. FEBS Lett. 268, 209.

Trifaró, J.-M., Lee, R.W.H., 1980. Morphological characteristics and stimulation secretion coupling in bovine adrenal chromaffin cell cultures. Neuroscience 5, 1533.

Trifaró, J.-M., Kenigsberg, R.L., Côté, A., Lee, R.W.H., Hikita, T., 1984. Adrenal paraneuron contractile proteins and stimulus secretion coupling. Can. J. Physiol. Pharmacol. 62, 493.

Trifaró, J.-M., Novas, M.L., Fournier, S., Rodríguez Del Castillo, A., 1989. Cellular and molecular mechanisms in hormone and neurotransmitter secretion. In: Velasco, M., Israel, A., Romero, E., Sila, H. (Eds.), Recent Advances in Pharmacology and Therapeutics. Elsevier, New York, NY, p. 15.

Vitale, M.L., Rodríguez Del Castillo, A., Tchakarov, L., Trifaró, J.-M., 1991. Cortical filamentous actin disassembly and scinderin redistribution during chromaffin cell stimulation precede exocytosis: a phenomenon not exhibited by gelsolin. J. Cell Biol. 113, 1057.

Vitale, M.L., Seward, E.P., Trifaró, J.-M., 1995. Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. Neuron 14, 353.

White, M.M., 1988. Forskolin alters acetylcholine receptor gating by a mechanism independent of adenylate cyclase activation. Mol. Pharmacol. 34, 427.

Wilson, S., 1988. Vasoactive intestinal peptide elevates cyclic AMP levels and potentiates secretion in bovine adrenal chromaffin cells. Neuropeptides 11, 17.