0026-895X/10/7804-734–744$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 78:734–744, 2010

Vol. 78, No. 4
66423/3626215
Printed in U.S.A.

Blockade by Nanomolar Resveratrol of Quantal Catecholamine Release in Chromaffin Cells

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Received May 16, 2010; accepted July 14, 2010

ABSTRACT

The cardiovascular protecting effects of resveratrol, an antioxidant polyphenol present in grapes and wine, have been attributed to its vasorelaxing effects and to its anti-inflammatory, antioxidant, and antiplatelet actions. Inhibition of adrenal catecholamine release has also been recently implicated in its cardioprotecting effects. Here, we have studied the effects of nanomolar concentrations of resveratrol on quantal single-vesicle catecholamine release in isolated bovine adrenal chromaffin cells. We have found that 30 to 300 nM concentrations of resveratrol blocked the acetylcholine (ACh) and high K^+-evoked quantal catecholamine release, amperometrically measured with a carbon fiber microelectrode. At these concentrations, resveratrol did not affect the whole-cell inward currents through nicotinic receptors or voltage-dependent sodium and calcium channels, neither the ACh- or K^+-elicited transients of cytosolic Ca^{2+}. Blockade by nanomolar resveratrol of secretion in ionomycin- or digitonin-treated cells suggests an intracellular site of action beyond Ca^{2+}-dependent exocytotic steps. The fact that nanomolar resveratrol augmented cGMP is consistent with the view that resveratrol could be blocking the quantal secretion of catecholamine through a nitric oxide-linked mechanism. Because this effect occurs at nanomolar concentrations, our data are relevant in the context of the low circulating levels of resveratrol found in moderate consumers of red wines, which could afford cardioprotection by mitigating the catecholamine surge occurring during stress.

Introduction

Increasing interest in the exploration of the pharmacological profile of resveratrol (3,4′,5-trihydroxystilbene), a compound present in grapes and wine, arose when it was associated to a putative cardiovascular protecting action. A number of large-scale epidemiological studies suggested that prolonged moderate consumption of red wine by Southern French and other Mediterranean populations was associated with a very low incidence of coronary heart disease, despite a high-fat diet, little exercise, and widespread smoking; this observation was coined as the French Paradox (Renaud and de Lorgeril, 1992). The French Paradox hypothesis generated much interest in the study of the effects of resveratrol on various cardiovascular risk-like parameters. For instance, vessels precontracted with phenylephrine or K^+ are relaxed by resveratrol (Chen and Pace-Asciak, 1996; Orallo et al., 2002). The fact that resveratrol activates soluble and particulate guanylate cyclase and augments vascular cGMP levels suggests the involvement of the L-arginine-NO pathway. Prevention of nitric oxide (NO) degradation by inhibiting NAD(P) oxidase has also been linked to resveratrol (Orallo et al., 2002). A direct protecting action on the heart through an NO-dependent mechanism has also been suggested previously (Hattori et al., 2002); such protection was associated to activation by resveratrol of a cGMP/protein kinase G path-

Supported in part by Ministerio de Ciencia e Innovación [Grant SAF2006-03589]; Agencia Laín Entralgo, Comunidad de Madrid [Grant NDE 07/9]; Fundación Centro de Investigación de Enfermedades Neurológicas, Instituto de Salud Carlos III [Grants PI016/09, PI080227]; Comunidad de Madrid [Grant S-SAL-0275-2006]; Instituto de Salud Carlos III [Grant RD 06/0026]; Ministerio de Sanidad y Consumo [Grant Fondo de Investigación Sanitaria PI061537]; and Consellería de Innovación e Industria de la Xunta de Galicia [Grants INCITE07PXI203039ES, INCITE08E1R203054ES, INCITE08CSA019203PR].

We acknowledge financial support from Centro de Estudios de América Latina-Universidad Autónoma Metropolitana-Banco de Santander.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.110.066423.

ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; IBMX, 3-isobutyl-1-methylxanthine; 75K^+, 75 mM K^+/low Na^+ solution; Q_{amp}, amperometric charge; MCN-A-343, 4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-*N*,*N*,*N*-trimethyl-2-butyn-1-aminium chloride; BayK8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-3-pyridinecarboxylic acid, methyl ester.

way (Xi et al., 2009). Anti-inflammatory, antioxidant, antiplatelet activity, and modulation of lipoprotein metabolism have also been associated with the cardiovascular protecting actions of resveratrol (Wu et al., 2001; Orallo et al., 2002; Bradamante et al., 2004). Another high-risk cardiovascular factor is excessive levels of circulating catecholamines as a result of hyperactivation of the sympathoadrenal axis. This occurs as a compensatory mechanism taking place during cardiac failure as a consequence of an acute myocardial infarction (Kaye et al., 1995; Lymperopoulos et al., 2007). In fact, blockers of β-adrenergic receptors are cardioprotective in this clinical setting and in myocardial infarction (Freemantle et al., 1999). It is therefore surprising that the effects of resveratrol on catecholamine release have been approached only in two recent studies. In the first study, Shinohara et al. (2007) analyzed the release of catecholamine in primary cultures of bovine adrenal chromaffin cells elicited by acetylcholine (ACh), high potassium concentrations (K^+), and veratridine; at micromolar concentrations, resveratrol blocked the responses to the three secretagogues. In a second study, Woo et al. (2008) explored the effects of resveratrol on catecholamine release from perfused rat adrenal glands stimulated with ACh, dimethylphenylpiperazinium, K^+, the muscarinic receptor agonist 4-[[[(3-chlorophenyl)amino]carbonyl] oxy]-*N*,*N*,*N*-trimethyl-2-butyn-1-aminium chloride (MCN-A-343), veratridine, cyclopiazonic acid, and 1,4-dihydro-2, 6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-3-pyridinecarboxylic acid, methyl ester (BayK8644), an activator of chromaffin cell L-type calcium channels (García et al., 1984). The authors found that very high resveratrol concentrations (10–100 μM) reduced approximately 30 to 50% of the secretory responses elicited by 2- to 4-min perfusion of distinct secretagogues. In most studies trying to decipher the mechanisms involved in the putative cardioprotective effects of resveratrol, including those of Shinohara et al. (2007) and Woo et al. (2008) on adrenal catecholamine release, high concentrations (up to 100 μM) of the polyphenol were used. Considering that only trace amounts of resveratrol have been found in plasma after its oral administration (Walle et al., 2004), that orally administered resveratrol to humans is quickly metabolized into sulfate and glucuronic acid resveratrol-derivatives (Walle et al., 2004); and that after long-term consumption of moderate amounts of red wine, blood levels of resveratrol range from 100 to 1000 nM (Bertelli, 2006), it follows that in vitro biological effects of micromolar resveratrol cannot explain the putative cardioprotective effects of moderate consumption of red wine. Here we have found that nanomolar concentrations of resveratrol caused a pronounced blockade of quantal catecholamine release from single bovine chromaffin cells stimulated with short ACh pulses. Because nanomolar resveratrol did not affect the whole-cell inward currents flowing through nicotinic receptors (I_{ACh}), sodium channels (I_{Na}), or calcium channels (I_{Ca}), as well as the cytosolic Ca^{2+} elevations ($[Ca^{2+}]_c$) produced by ACh or K^+, we favor the possibility that resveratrol is directly acting on the last Ca^{2+}-independent steps of fusion pore formation and exocytosis through a mechanism similar to that produced by NO and NO donors, also described to take place in bovine chromaffin cells (Machado et al., 2000).

Materials and Methods

Isolation and Culture of Adrenal Medulla Chromaffin Cells. All experiments have been carried out in accordance with the Declaration of Helsinki and with the guide for care and use of laboratory animals as adopted and promulgated by the Universidad Autónoma de Madrid. Bovine adrenal medulla chromaffin cells were isolated according to standard methods with some modifications (Moro et al., 1990). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. For patch-clamp studies and single-cell measurement of catecholamine release, cells were plated on 13-mm diameter glass coverslips at low density (7.5×10^4 cells/coverslip). To study the changes of $[Ca]_c$, cells were plated at a density of 5×10^5 cells/well in 25-mm six-well plates.

Amperometric Recordings of Single Vesicle Quantal Catecholamine Release. Amperometry was chosen to measure catecholamine release at the single cell level (Wightman et al., 1991). Electrodes were built as described previously (Wightman et al., 1991). The amperometer was homemade (Universidad Autónoma de Madrid workshop) and was connected to an interface (PowerLab/4SP; ADInstruments, New Zealand) that digitized the signal at 10 kHz sending it to an Apple Macintosh Power PC computer that displayed it within the Chart V. 4.2 software (ADInstruments, Dunedin, New Zealand). A 730-mV potential was applied to the electrode with respect to an AgCl ground electrode. The electrodes were calibrated according to good amperometric practices (Machado et al., 2008) by perfusing 50 μM norepinephrine dissolved in standard Tyrode's solution and measuring the current elicited; only electrodes that yielded a current between 200 and 400 pA were used. At micromolar concentrations, resveratrol underwent some oxidation by the current passing through the carbon fiber microelectrode tip. This could lead to erroneous conclusions on the effects of resveratrol on the oxidation of catecholamine being released by the different secretagogues. To ascertain the extent of this interference, we recorded the amperometric current generated by a Tyrode's solution containing 50 μM norepinephrine. We observed that after 5-min exposure of the carbon fiber to 300 nM resveratrol, the current generated by norepinephrine decreased by 29 ± 4.4% (n = 5 microelectrodes). Such interference amounted to 24.9 ± 7.2% sensitivity loss after 5-min exposure to 100 nM resveratrol (n = 7 electrodes). In the light of these data, and to minimize interference with amperometric detection of catecholamine release elicited by the various secretagogues, experiments were performed with resveratrol concentrations ranging between 30 and 300 nM resveratrol. Appropriate catecholamine standards were always used to test the variations in the electrode sensitivity. The coverslips were mounted in a chamber on a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) used to localize the target cell, which was continuously superfused by means of a five-way superfusion system with a common outlet driven by electrically controlled valves, with a Tyrode's solution composed of 137 mM NaCl, 1 mM $MgCl_2$, 5.3 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3, NaOH. The high K^+ solutions were prepared by replacing equiosmolar concentrations of NaCl with KCl. At the time of experiment performance, proper amounts of drug stock solutions were freshly dissolved into the Tyrode's solution.

Measurements of Whole-Cell Inward Currents. Ca^{2+} (I_{Ca}), Na^+ (I_{Na}), and ACh (I_{ACh}) currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Whole-cell recordings were made with fire-polished electrodes (resistance of 2–5 MΩ when filled with the standard intracellular solutions) mounted on the head stage of an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany), allowing cancellation of capacitative transients and compensation of series resistance. Data were acquired with a sample frequency ranging between 5 and 10 kHz and filtered at 1 to 2 kHz. Recording traces with leak currents >100 pA or series resistance >20 MΩ were discarded. Data acquisition and analysis

were performed using PULSE programs (HEKA). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Eclipse T2000 inverted microscope. During the preparation of the seal with the patch pipette, the chamber contained a control Tyrode's solution plus 5 μM tetrodotoxin, pH 7.4 (no tetrodotoxin was added when measuring I_{Na} or I_{ACh}). Once the patch membrane was ruptured and the whole-cell configuration of the patch-clamp technique had been established, the cell was locally, rapidly, and constantly superfused with an extracellular solution of similar composition to the chamber solution but containing nominally 0 mM Ca^{2+} to measure I_{Na} or 10 mM Ca^{2+} to record I_{Ca} (see *Results* for specific experimental protocols). For ionic current recordings, cells were dialyzed with an intracellular solution containing 100 mM CsCl, 14 mM EGTA, 20 mM TEA-Cl, 10 mM NaCl, 5 mM Mg-ATP, 0.3 mM Na-GTP, and 20 mM HEPES/CsOH, pH 7.3. The external solutions were rapidly exchanged using electronically driven miniature solenoid valves coupled to a multibarrel concentration-clamp device, the common outlet of which was placed within 100 μm of the cell to be patched. The flow rate was 1 ml/min and regulated by gravity. Experiments were performed at room temperature (23–25°C).

Measurements of Changes in the Cytosolic Ca^{2+} Concentrations. The setup for fluorescence recordings was composed of a Nikon Diaphot 200 microscope and a Bio-Rad confocal unit (Bio-Rad Laboratories, Hemel Hempstead, UK) equipped with an oil immersion objective (Nikon 60× Plan Apo; numerical aperture, 1.4). Chromaffin cells were loaded with 10 μM concentration of the calcium probe fluo-4-acetoxymethyl ester for 45 min at 37°C as described previously (de Diego et al., 2008). Sampling rate was 1 s during stimuli and 5 s for periods in between stimuli. A Tyrode external solution was used of the same composition as the one described above; concentrations of drugs added to this solution are indicated in the text.

cGMP Assay. Cells were plated onto 24-well dishes at a density of 5×10^5 cells/well and maintained at 37°C in 5% CO_2/95% air. Four days after isolation, chromaffin cells were preincubated with Tyrode's solution at 37° for 30 min. Then they were stimulated with agents in 0.3 ml of Tyrode's solution (with or without IBMX) for the indicated times. The reaction was stopped by the addition of 1% Triton X-100. Cells were scraped off of the wells, and the cGMP content was measured with a commercially available cGMP enzyme immunoassay kit (Sigma-Aldrich, St. Louis, MO) according to the instructions supplied by the manufacturer.

Reagents and Solutions. All reagents for the solutions were purchased from Sigma-Aldrich and Panreac Quimica (Barcelona, Spain). Dulbecco's modified Eagle's medium and penicillin-streptomycin were from Gibco (Carlsbad, CA), and bovine serum was from PAA Laboratories GmbH (Pasching, Austria). Fluo-4 was from Invitrogen (Carlsbad, CA).

Data Analysis and Statistics. Data analysis was carried out on a personal computer using Excel (Microsoft, Redmond, WA) and IgorPro (Wavemetrics, Lake Oswego OR). Amperometric charge (Q_{amp}) was calculated by integrating the amperometric current over time during the stimulus duration with a macro written in IgorPro. The number of spikes greater than 5 pA was manually counted on an extended graph displayed in the computer screen. A ruler was drawn at 5 pA, and spikes going above the threshold amplitude were considered. Differences between means of group data fitting a normal distribution were assessed by using either analysis of variance or Kruskal-Wallis test for comparison among multiple groups or Student's t test for comparison between two groups. $p < 0.05$ was taken as the limit of significance.

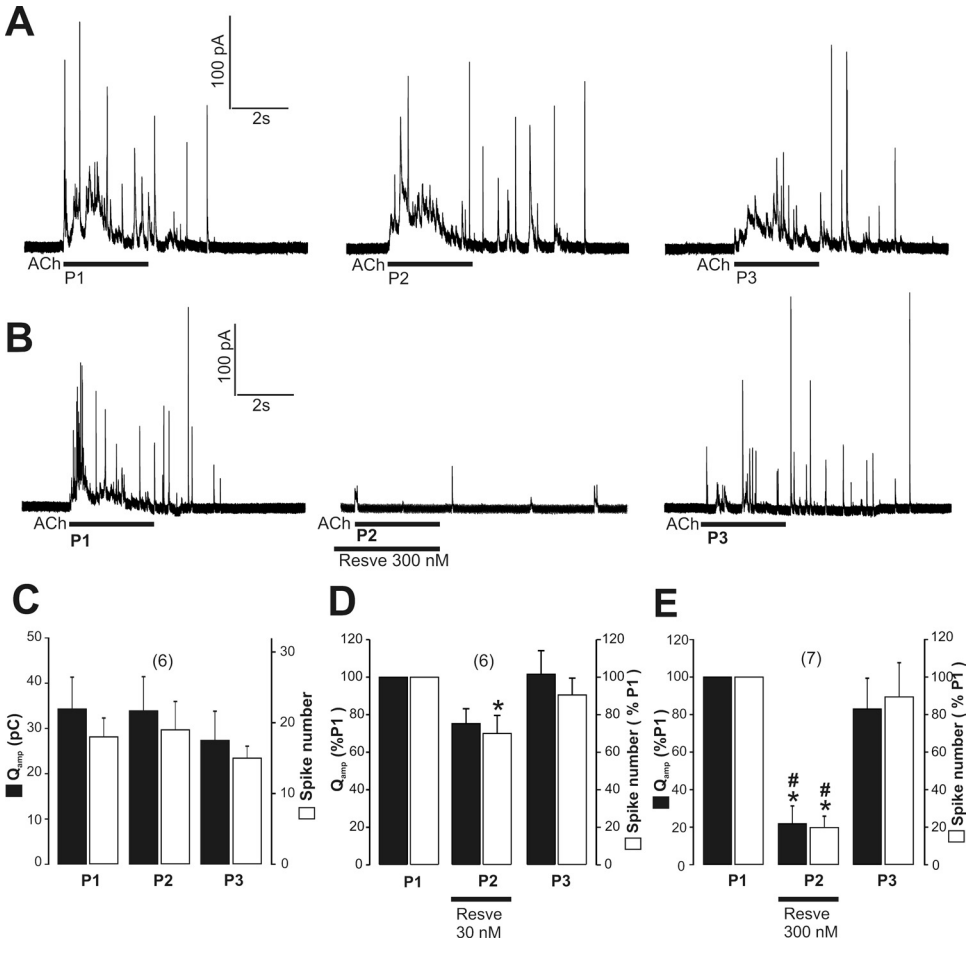

Fig. 1. Blockade of ACh-evoked quantal catecholamine release by resveratrol. A, example traces obtained in the same cell, generated by three sequential ACh pulses (P1, P2, and P3) applied at 5-min intervals. The pulses were applied with a Tyrode's solution containing 300 μM ACh during a 3-s period. B, example traces obtained in the same cell showing a sandwich-type experiment consisting of an initial control ACh pulse (P1); then resveratrol was perifused for 4 min before and during application of P2. Finally, after 5-min washout, a third recovery pulse was applied (P3). C, pooled data from experiments performed with protocol A, showing the integrated cumulative secretion per pulse (Q_{amp}, left ordinate) and spike number per pulse (right ordinate). D and E, averaged normalized secretion obtained with experiments performed with protocol B; data were normalized within each individual cell as a percentage of P1 both for cumulative secretion (left ordinate) and spike number (right ordinate). Data are means ± S.E. of the number of cells shown in parentheses from one to three different cultures. *, $p < 0.05$ compared with P1; #, $p < 0.05$ compared with P3.

Results

Quantal Catecholamine Release Responses Elicited by ACh: Effects of Resveratrol. To measure the effects of resveratrol on Ca^{2+}-dependent exocytosis triggered by various stimuli, we recoursed to single-cell recording of individual amperometric spikes, which are the result of quantal secretion of the catecholamine contents of individual chromaffin vesicles (Wightman et al., 1991). The experiment began with the continuous local perifusion of the selected cell with a Tyrode's solution containing 2 mM Ca^{2+} for approximately 1 min to obtain a stable baseline. Usually, no spontaneous amperometric spikes were detected. After baseline stabilization, the cell was challenged three successive times with 3-s duration pulses (P1, P2, P3), given with a solution containing 300 μM ACh at 5-min intervals; this evokes sharp reproducible secretory responses. Figure 1A shows three spike traces obtained in an example cell. ACh promptly elicited a burst of quantal secretory spikes that lasted along the ACh pulse duration. In some cells, their secretory activity ceased before stopping ACh perifusion, whereas in others, such secretory activity lasted 1 to 2 s after ACh removal. In this particular cell, P1 generated a trace with pronounced baseline elevation as a result of large secretory activity and spike overlapping. This pattern was similar during P2 and P3. In other cells, baseline elevation was less pronounced (as in P1 of Fig. 1B) or was even absent (as in P3 of Fig. 1B). This sequential stimulation with ACh produced quite reproducible secretory responses when applied within the same cell, as illustrated in the bar graph of Fig. 1C; total cumulative secretion (Q_{amp}) and number of spikes were similar in P1 and P2 and underwent a 20% decrease in P3, with respect to P1 and P2. This design permitted the exploration of the effects on secretion of different concentrations of resveratrol adopting a sandwich-type protocol, as that displayed in Fig. 1B in which the perifusion of 300 nM resveratrol practically abolished the ACh secretory response. This drastic effect was readily and fully reversible after washout of resveratrol, as indicated by the healthy response of Fig. 1B, P3. Averaged results are given in the bar graph of Fig. 1E, indicating that 300 nM resveratrol inhibited by 80% both Q_{amp} and spike number, with a practical full recovery of the response after 5 min of resveratrol washout. At 30 nM, resveratrol caused 23 ± 8.7% inhibition of spike number (Fig. 1D).

Inhibition by Resveratrol of ACh-Evoked Whole-Cell Inward Currents (I_{Ach}). At first glance, an obvious mechanism involved in the blockade by resveratrol of ACh-elicited secretion could be the inhibition of nicotinic receptors for ACh (nAChRs). Shinohara et al. (2007) have shown that resveratrol blocks I_{ACh} in oocytes expressing $\alpha3\beta4$ nAChRs with an IC_{50} of 25.9 μM. We also tested the effects of resveratrol on I_{ACh} but on native $\alpha3\beta4$ receptors of bovine chromaffin cells, voltage-clamped at −80 mV. Inward currents were evoked by repeated pulses of ACh. Such pulses (300 μM, 0.5 s) produced peak I_{ACh} that are highly reproducible when given at 30-s intervals for at least 15 min (data not shown). Figure 2A shows the time course of I_{ACh} generated by sequential ACh pulses applied to an example cell. Note the initial current amplitude that was quite stable at approximately 1.7 nA. In other cells, the submicromolar concentrations of resveratrol that caused a drastic inhibition of secretion (i.e., 30–300 nM), however, did not touch I_{ACh} (data not shown). Much higher concentrations of the compound caused a quick depression of I_{ACh} that was gradually reversed upon its washout. The original I_{ACh} traces shown in Fig. 2B were taken from the points labeled with small letters in Fig. 2A; it seemed that at 30 or 100 μM, resveratrol did not change the current kinetics. Figure 2C displays a concentration-response curve for the blockade of I_{ACh} by resveratrol, with an IC_{50} of 56 μM, close to that found in oocytes expressing $\alpha3\beta4$ nAChRs, namely 25.9 μM (Shinohara et al., 2007). This indicates that resveratrol is approximately 600-fold less potent to block I_{ACh} compared with its ability to inhibit ACh-evoked quantal secretion (Fig. 1). As a consequence, these inhibitory effects of nanomolar concentrations of resveratrol cannot be

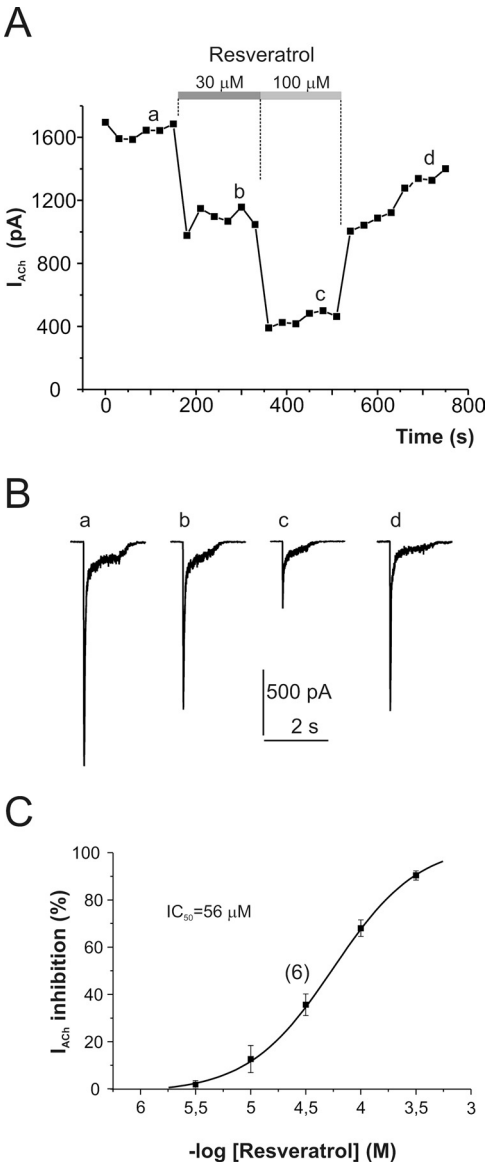

Fig. 2. Inhibition by resveratrol of the whole-cell inward currents elicited by ACh (I_{ACh}). These experiments were performed under the whole-cell configuration of the patch-clamp technique in cells voltage-clamped at −80 mV. A, time course of I_{ACh} amplitude (ordinate) in an example cell that was stimulated with sequential ACh pulses (300 μM ACh, 0.5 s) given at 30-s intervals. Resveratrol was applied at the concentrations and during the time periods marked by the top horizontal bars. B, original current traces obtained from the points shown in A indicated by lowercase letters. C, concentration-response curve indicating I_{ACh} blockade by increasing resveratrol concentrations; data are means ± S.E. of six cells.

attributed to its capacity to block nAChR currents at much higher concentrations.

Inhibition by Resveratrol of Whole-Cell Inward Currents through I_{Na}. Chromaffin cells are depolarized by ACh that produces Na^+-dependent action potentials that will give rise to Ca^{2+}-dependent release of catecholamines (de Diego et al., 2008; de Diego, 2010). The possibility that resveratrol was acting on Na^+ channels to block action potentials and secretion elicited by ACh was explored by measuring I_{Na} in voltage-clamped cells subjected to repeated depolarizing pulses to 0 mV, given from a holding potential of −80 mV at 10-s intervals. Under these conditions, I_{Na} amplitude remained stable for at least a 10-min period; I_{Na} was fully blocked by 1 μM tetrodotoxin (data not shown). Figure 3A shows the time course of I_{Na} amplitude (peak current) in an example voltage-clamped cell. The initial I_{Na} amplitude (approximately 1.2 nA) was mildly decreased in a step-wise manner by increasing concentrations of resveratrol (3–100 μM). However, at 100 to 300 nM, resveratrol did not affect I_{Na} (data not shown). Figure 3B shows original I_{Na} traces taken from Fig. 3A at the points identified with lowercase letters; at 10 to 100 μM, resveratrol did not change the current kinetics. Figure 3C shows a concentration-response curve for the inhibitory effects of resveratrol on I_{Na}. Threshold blockade was achieved at 10 μM, whereas maximal blockade at 100 μM was only 25%. An IC_{50} for this partial blockade was roughly estimated to be 25 μM. Measuring $^{22}Na^+$ uptake elicited by ACh, Shinohara et al. (2007) found that resveratrol caused a blockade with an IC_{50} of 20.4 μM.

Inhibition by Resveratrol of the K^+-Induced Quantal Release of Catecholamine. Depolarization of chromaffin cells by high K^+ concentrations opens voltage-dependent calcium channels to enhance Ca^{2+} entry and stimulate catecholamine release (Douglas and Rubin, 1963). Resveratrol could be blocking those channels and Ca^{2+} entry, thereby causing inhibition of secretion. Thus, we decided to study the effects of nanomolar resveratrol on potassium-evoked quantal secretion. At 75 mM, K^+ shifts the membrane potential of bovine chromaffin cells to near 0 (Orozco et al., 2006), which will recruit all Ca^{2+} channel subtypes, L, N, and P/Q, expressed by these cells (García et al., 2006); thus, we chose this concentration of K^+ to trigger the release of catecholamines and study the effects of resveratrol on this response. Figure 4A shows three secretory spike traces obtained from the same example cell that was sequentially challenged with 10-s duration pulses given at 5-min intervals with a solution containing 75 mM K^+/low Na^+ (75K^+). As in the case of ACh (Fig. 1A), K^+ rapidly produced a burst of secretory spikes that was quite reproducible during the three challenges. Unlike ACh, more cells exhibited a milder baseline elevation at the beginning of the traces (Fig. 4A) or no elevation at all (Fig. 4B). This could explain that more individual secretory spikes were counted in the K^+ traces (approximately 40–50 per stimulus; Fig. 4C) compared with ACh traces (approxi-

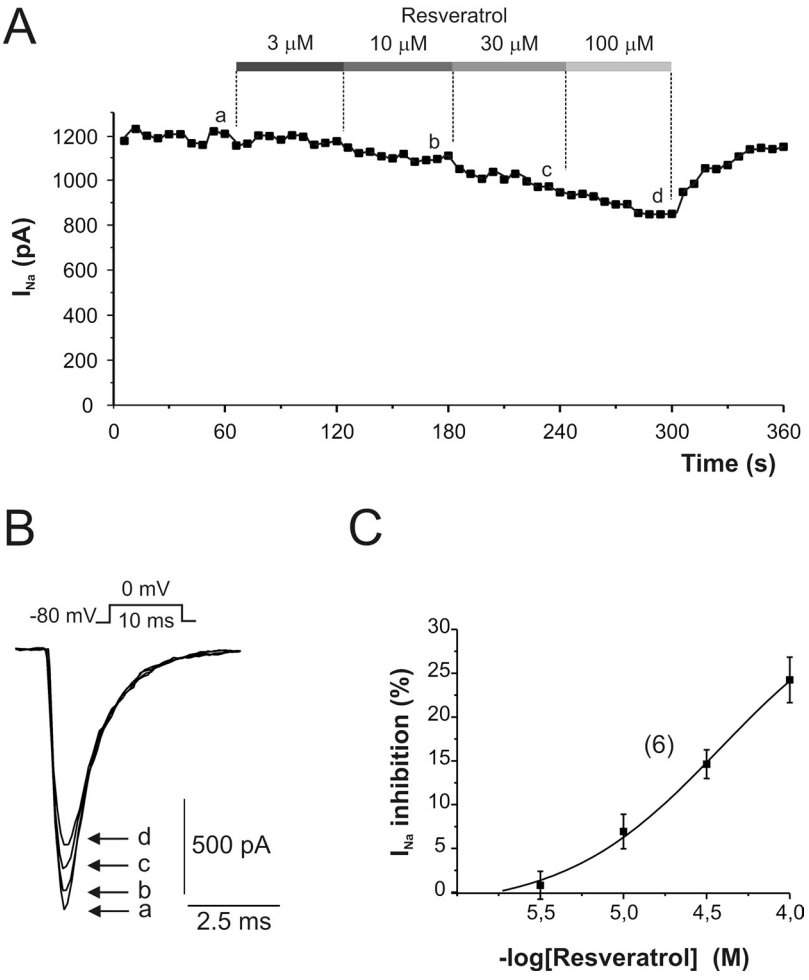

Fig. 3. Partial blockade by resveratrol of the whole-cell inward sodium current (I_{Na}) through voltage-dependent sodium channels. Cells were voltage-clamped at holding potential of −80 mV. I_{Na} was elicited by successive voltage depolarizing steps to 0 mV given at 10-s intervals. A, time course of I_{Na} amplitude (peak current) in an example cell before (control) and upon cell perifusion with increasing concentrations of resveratrol, as indicated by the top horizontal bars. B, original I_{Na} traces taken from the points labeled with lowercase letters in the time course curve shown in A. C, concentration-response curve of the inhibitory effect of resveratrol on I_{Na}; data are means ± S.E. of six cells.

mately 15–25 per stimulus; Fig. 1C). Measurements of integrated secretion per stimulus (Q_{amp}) and spike number in control conditions produced similar responses, as illustrated in the bar graph of Fig. 4C. This permitted the exploration of the effects of resveratrol on secretion using a sandwich type experimental protocol. Figure 4B shows an example cell in which 300 nM resveratrol caused a drastic inhibition of secretory events during P2, compared with the initial P1 response in the absence of the compound. Upon resveratrol washout, the cell secretory activity greatly recovered. Averaged normalized results indicated a concentration-dependent effect of resveratrol to block quantal secretion. Thus, at 30 nM, blockade was 25% (D), at 100 nM, 40% (E), and at 300 nM, 65% (F). Cumulative secretion and spike number were reduced to a similar extent.

Effects of Resveratrol on the Whole-Cell Inward Calcium Channel Currents through I_{Ca}. As referenced above, the secretory response elicited by K^+ pulses is due to augmented Ca^{2+} entry through calcium channels. Thus, resveratrol could cause the blockade of K^+-elicited secretion by inhibiting the various subtypes of voltage-dependent calcium channels expressed by bovine chromaffin cells (García et al., 2006). This possibility was tested by measuring the whole-cell inward currents elicited by 50-ms test depolarizing pulses applied to cells voltage-clamped at −80 mV using 10 mM Ca^{2+} as charge carrier.

Figure 5A shows the time course of I_{Ca} amplitude in an example cell being challenged with repeated test depolarizing pulses. The initial peak current was at approximately 900 pA. At the nanomolar concentrations used to study its effects on quantal release, resveratrol did not affect I_{Ca} (data not shown). Resveratrol (10–30 μM affected neither the current amplitude nor its kinetics (inset). Figure 5B shows I-V curves obtained before and in the presence of 30 μM resveratrol.

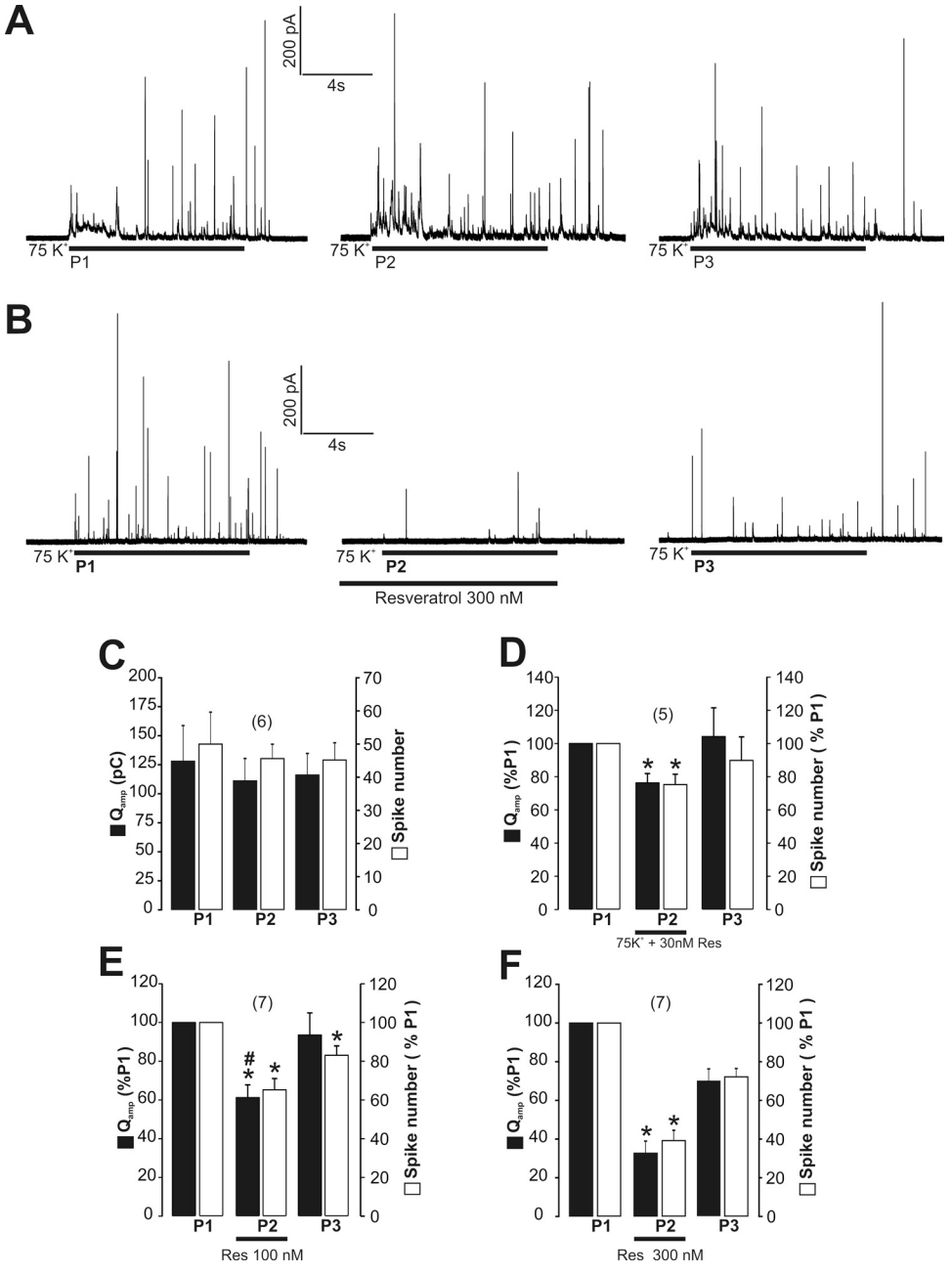

Fig. 4. Resveratrol inhibits the quantal catecholamine release elicited by K^+. A, control example cell that was challenged with three sequential 10-s pulses (P1, P2, and P3) of a 75 mM K^+/low Na^+ solution (75K^+), given at 5-min intervals. B, experimental example cell that was challenged with three K^+ pulses; resveratrol was present 4 min before and during P2. C, pooled data from control experiments performed in cells using the protocol described in A. D, E, and F show pooled results obtained with experiments performed with protocol B and increasing concentrations of resveratrol on total secretion per pulse (left ordinate) and spike number per pulse (right ordinate), normalized as the percentage of P1. Data are means± S.E. of the number of cells shown in parentheses from two to three different cultures. *, $p < 0.05$ with respect to P1. #, $p < 0.05$ with respect to P3.

There was no shift or inhibition of I_{Ca} in the presence of these high concentrations of the compound. Therefore, it seems that resveratrol is inhibiting the K^+ and the ACh-elicited exocytotic responses by a mechanism unrelated to Ca^{2+} entry through calcium channels. $^{45}Ca^{2+}$ influx into bovine chromaffin cells stimulated with ACh was inhibited by resveratrol with an IC_{50} of 62.8 μM (Shinohara et al., 2007).

Effects of Resveratrol on the Elevations of $[Ca^{2+}]_c$ Elicited by ACh or K^+ Pulses. In chromaffin cells, exocytosis has an absolute requirement for Ca^{2+}. Hence, we explored the possibility that resveratrol could affect the $[Ca^{2+}]_c$ elevations elicited upon challenging of the cells with ACh or K^+ pulses, despite the fact that at nanomolar concentrations, the compound did not block either nAChR channels or voltage-dependent sodium and calcium channels. Figure 6A shows the $[Ca^{2+}]_c$ traces obtained in an example cell challenged with ACh pulses (300 μM, 10 s) given at 5-min intervals. Four minutes before and during P2, 300 nM resveratrol was introduced. The compound produced by itself a rapid elevation of the $[Ca^{2+}]_c$ that decreased to baseline levels after 2.5 min of continued cell perifusion with resveratrol before P2 was applied. The presence of the compound did not alter the ACh-elicited $[Ca^{2+}]_c$ transient. Pooled results of these experiments are given in Fig. 6B; note that at 300 nM, resveratrol itself produced a $[Ca^{2+}]_c$ elevation with an amplitude that was 50% of that of P1. However, the compound did not significantly modify the ACh-evoked $[Ca^{2+}]_c$ elevation. The nature of this transient $[Ca^{2+}]_c$ elevation elicited by nanomolar resveratrol is presently being studied in our laboratory. Similar experiments were done using three sequential K^+ challenges (75 mM K^+, 10 s) given at 5-min intervals, as exemplified in the fluo-4-loaded cells of Fig. 6B. Averaged data are given in Fig. 6, C and D. Resveratrol itself produced a $[Ca^{2+}]_c$ transient that was approximately 40 to 50% of P1. Note that resveratrol caused approximately 20% decrease of the ACh- and K^+-evoked $[Ca^{2+}]_c$ elevation that did not reach the level of statistical significance.

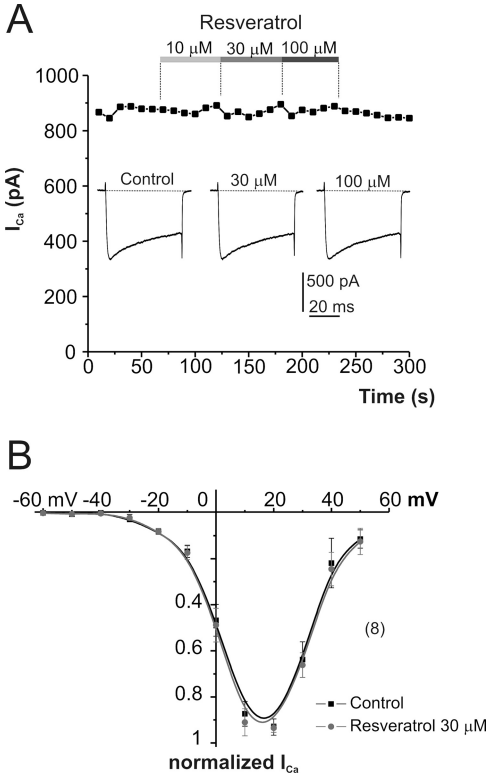

Fig. 5. Resveratrol does not affect the whole-cell inward calcium current through voltage-dependent calcium channels (I_{Ca}). Cells were voltage-clamped at a holding potential of −80 mV. I_{Ca} was elicited by sequential depolarizing pulses given at 10-s intervals. A, time course of I_{Ca} amplitude (peak current) in an example cell, elicited by test pulses to 0 mV, before (control) and upon cell perifusion with increasing concentrations of resveratrol, as indicated by the top horizontal bars. Inset, original I_{Ca} traces taken from the time course curve. B, current-voltage relationship before (control) and during cell perifusion with 30 μM resveratrol. Data are means ± S.E. of eight cells from two different cultures.

Resveratrol Inhibited the Quantal Release of Catecholamine Triggered by Ionomycin in Intact Cells or by Ca^{2+} in Digitonin-Permeabilized Cells. Because plasmalemmal ion channels seemed not to be involved in the mechanism underlying the drastic blockade of exocytosis by resveratrol, we further looked at a possible intracellular site of action of the compound by studying its effects on quantal catecholamine release responses triggered under experimental conditions that bypass plasmalemmal ion channels. Two such conditions were used, namely ionomycin and digitonin. Ionomycin is known to cause the release of catecholamines by a Ca^{2+}-dependent mechanism but induces direct Ca^{2+} entry into chromaffin cells without the intervention of Ca^{2+} channels (Carvalho et al., 1982). Figure 7A shows the continuous secretory activity of a cell that is being perifused with 10 μM ionomycin. This pronounced activity remains constant for as long as a 10-min period, although at 8 to 10 min, secretion tended to decline (see averaged data in Fig. 7D). In addition, the example cells of Fig. 7, B and C, were continuously perifused with ionomycin. However, during the 2- to 4- and 6- to 8-min periods, 100 nM (B) or 300 nM (C) resveratrol was given on top of the ionophore. Secretion was rapidly and nearly fully inhibited during the time periods of resveratrol treatment; some spikes were visible during resveratrol treatment, particularly during the second treatment period. Averaged data on these inhibitory effects of resveratrol are given in Fig. 7, E and F. The integral secretion in 2 min (Q_{amp}), and spike number was decreased by 70% with 100 nM (E) and 80 to 90% by 300 nM resveratrol (F). The second condition consisted in triggering quantal catecholamine release from digitonin-permeabilized cells. To perform this experiment, cells were first washed in a Ca^{2+}-free Krebs-HEPES solution and resuspended in an intracellular solution of the following composition: 140 mM KCl, 3 mM $MgCl_2$, 10 mM NaCl, 2 mM EGTA, 20 mM HEPES, 2 mM K_2-ATP, and 1 mM KH_2PO_4, pH 7.1. Cells were placed in the microchamber and perifused with this same solution containing 2 μM digitonin. Figure 8A shows an example amperometric spike record taken from a cell that was perifused with the intracellular solution deprived of Ca^{2+} and containing 2 μM digitonin. Secretion was triggered by introducing 30 to 50 μM free $[Ca^{2+}]$. After a 7-s delay, the cell begun to fire amperometric secretory spikes that were initiated with a burst followed by continuous secretory activity with progressive decline at the end of the trace. In the trace shown in Fig. 8B, Ca^{2+} was given in the presence of 300 nM resveratrol; an initial short-lasting spike burst was followed by spikes having lower amplitude and frequency compared with those obtained in the control cell (Fig. 8A). Cumulative secretion (Q_{amp}) and spike number were calculated at 30-s intervals in each individual cell; they were averaged and

plotted in Fig. 8, C and D, showing that resveratrol drastically diminished both parameters.

Effects of Resveratrol on the Levels of cGMP. NO donors cause a pronounced decrease of the amplitude of amperometric events and a slowing down of their kinetics through a mechanism dependent on guanylate cyclase and cGMP (Machado et al., 2000). We therefore measured the levels of this cyclic nucleotide under conditions similar to those used to monitor the effects of resveratrol on quantal secretion evoked by ACh or K^+. Figure 9A shows that control cells had basal levels of cGMP of 1.5 fmol/10^6 cells. At 30 nM, resveratrol did not augment those levels. However, at 100 and 300 nM, resveratrol enhanced cGMP to approximately 2 and 3 fmol/10^6 cells. In cells incubated with phosphodiester-

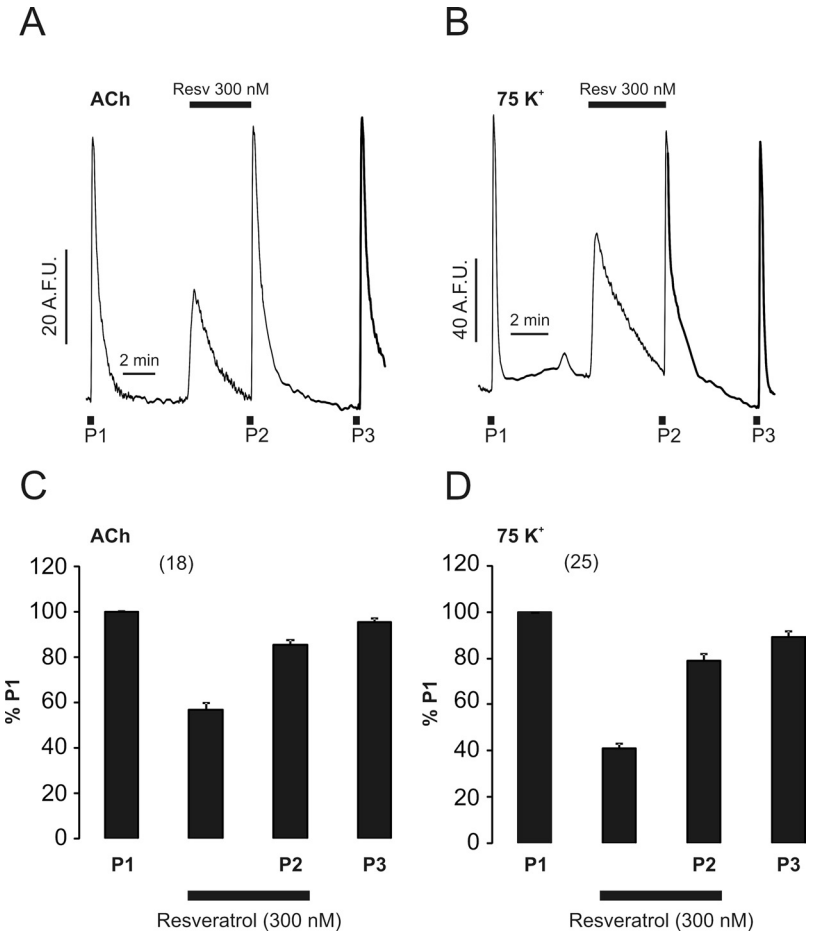

Fig. 6. Resveratrol does not affect the elevations of the cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) elicited by ACh or K^+ pulses. A protocol similar to that used to study exocytosis at the single-cell level was used here to analyze the effects of 300 nM resveratrol on the $[Ca^{2+}]_c$ transients elicited by 300 μM ACh or 75 mM K^+ pulses (10-s given at 5-min intervals, P1, P2, and P3) in fluo-4-loaded cells. A, example cell challenged with three sequential ACh pulses. B, averaged results on the $[Ca^{2+}]_c$ elevations elicited by ACh pulses in the absence and the presence (P2) of resveratrol; data are means ± S.E. of the number of experiments and the number of cultures shown in parentheses. C, example cell challenged with the three sequential 75K^+ pulses; note the transient elevation of $[Ca^{2+}]_c$ elicited by 300 nM resveratrol itself. D, means ± S.E. of the number of cells are shown in parentheses.

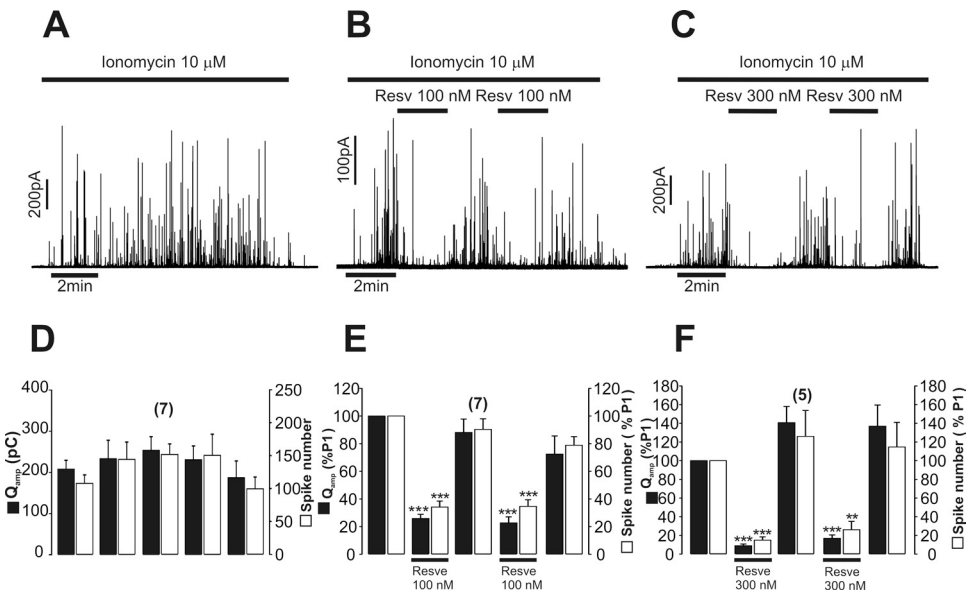

Fig. 7. Quantal catecholamine release elicited by ionomycin was drastically inhibited by resveratrol. A, this cell was continuously perifused with ionomycin, as indicated by the top horizontal bar; soon after ionophore perifusion, the cell fired continuous amperometric spike events. B, the cell was continuously perifused with ionomycin, as indicated by the top horizontal bar; resveratrol was given as indicated by the two horizontal bars. C, cumulative secretion, black columns (left ordinate), and spike number (right ordinate) estimated at 2-min periods (abscissa) in seven cells subjected to protocol A. E and F, pooled results on the effects of resveratrol, tested according to protocols B and C, normalized as a percentage of P1, on total cumulative secretion per pulse (left ordinate) and spike number (right ordinate). Data are means ± S.E. of the number of cells shown in parentheses, taken from two different cultures. **, $p < 0.01$, ***, $p < 0.001$ with respect to their respective previous control in the absence of resveratrol; ##, $p < 0.01$ with respect to their respective previous control in the absence of resveratrol.

ase inhibitor IBMX (Fig. 9B), basal cGMP levels increased to 3 fmol/10^6 cells. This time, 30 nM resveratrol augmented those levels to 4.5 fmol/10^6 cells. Further increase was seen at 100 and 300 nM resveratrol (i.e., approximately 6 fmol/10^6 cells).

Discussion

Central to this study is the observation that nanomolar concentrations of resveratrol block the quantal single-vesicle release of catecholamine from individual chromaffin cells stimulated with short ACh or K^+ pulses, as well as in ionomycin- or digitonin-treated cells. A priori, plasmalemmal nicotinic receptors, sodium channels, or calcium channels can be discarded to explain this secretion blockade because 100 to 300 nM resveratrol did not affect the ion currents flowing through those channels (Figs. 2, 3, and 5). This conclusion differs from that of Shinohara et al. (2007), suggesting that resveratrol blocks bulk catecholamine release in bovine chromaffin cells incubated during 2 to 4 min with various secretagogues. Because they found a blockade also of $^{22}Na^+$ and $^{45}Ca^{2+}$ uptake into cells incubated with veratridine, nicotine, or K^+, they suggested that secretion blockade was a result of sodium channel and calcium-channel blockade. Several differences could explain this discrepancy. First, we recorded I_{ACh}, I_{Na}, and I_{Ca}, which is a more direct and reliable means of testing a drug effect on ion channel activity, at a much higher time resolution (milliseconds compared with minutes). Second, measurement of single-vesicle quantal catecholamine release in an isolated cell has much higher time resolution than secretion measured in minutes. Third, our stimulation pattern is closer to physiology (time range of milliseconds to few seconds). But the main difference between the study of Shinohara et al. (2007) and ours is the potency of resveratrol to block secretion; we found an extraordinary efficacy to block single-cell quantal catecholamine release at nanomolar concentrations. In contrast, Shinohara et al. (2007) found that resveratrol inhibits bulk secretion in cell populations stimulated with ACh with an IC_{50} of 20.4 μM. This striking difference may be due to measurement of quantal single-vesicle release in our study, with a time resolution in the more physiological millisecond time range.

In their study on perfused rat adrenals, Woo et al. (2008) also found that high resveratrol concentrations (10–100 μM) block bulk catecholamine release measured in 4-min periods. On the basis of experiments performed with BayK8644, these authors also suggest that resveratrol could be blocking the L-subtype of voltage-dependent calcium channels to inhibit

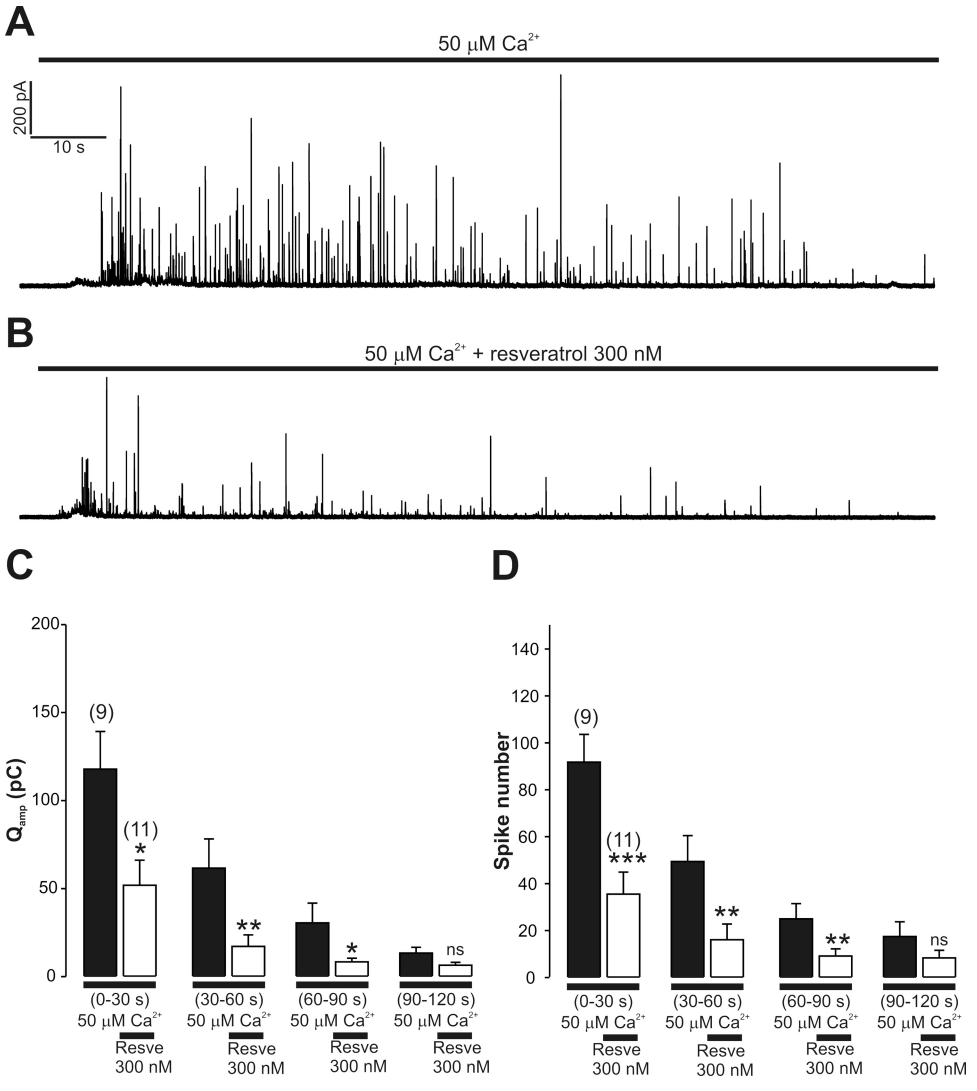

Fig. 8. Decrease of quantal secretion elicited by 50 μM Ca^{2+} in digitonin-permeabilized chromaffin cells. After 4 min of cell perifusion with an intracellular solution deprived of Ca^{2+} (see text), 50 μM free-Ca^{2+} buffer was applied for 2 min with or without 300 nM resveratrol. A, quantal secretion spikes elicited in control cells by digitonin treatment; B, quantal secretion in the presence of 300 nM resveratrol. Pooled results are shown in C (integrated charge, Q_{amp}) and D (spike number). The 2-min period in the presence of Ca^{2+} was divided in 30-s chunks to better appreciate the gradual decline of secretion, which was significantly smaller with resveratrol in all but the last period. Data are means ± S.E. of the number of cells shown in parentheses from three different cultures. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's t test.

secretion. Our results on direct I_{Ca} measurements show that even at extremely high concentrations (i.e., 100 μM, Fig. 5A), resveratrol did not affect I_{Ca}. The question now arises as to which mechanism could be involved in the blockade by nanomolar resveratrol of quantal catecholamine release.

We did not find a blocking effect of nanomolar concentrations of resveratrol on I_{ACh}, I_{Na}, and I_{Ca}. This suggests an intracellular site of action to explain the blocking effects of resveratrol on single-vesicle quantal catecholamine release. The intracellular location of such a site is strongly supported by the experiment with ionomycin, a Ca^{2+}-selective ionophore that causes Ca^{2+}-dependent catecholamine release from adrenal glands (Carvalho et al., 1982). This effect is not linked to plasmalemmal ion channels because the ionophore is incorporated into membranes and promotes Ca^{2+} movement according to their electrochemical gradients (Carvalho et al., 1982). The experiment on Ca^{2+}-evoked catecholamine release from digitonin-permeabilized chromaffin cells (Fig. 8), in which resveratrol elicited a pronounced blockade, also supports an intracellular site of action for the compound. Because resveratrol did not affect $[Ca^{2+}]_c$ elevations elicited by ACh and K^+, it seems that its effect must be linked to a Ca^{2+}-independent step in the chain of events leading to membrane fusion and pore formation at the very last steps of exocytosis.

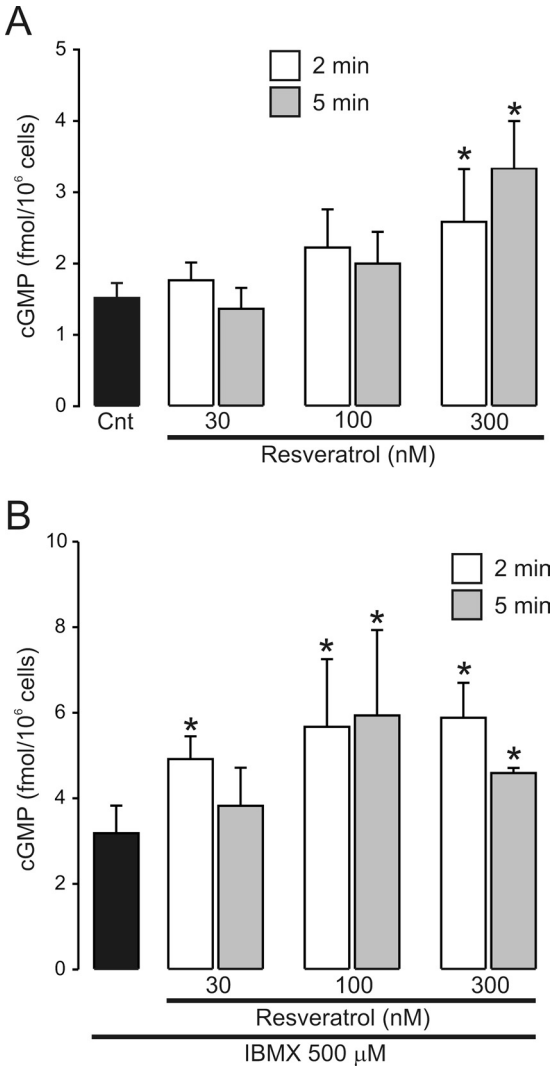

Fig. 9. cGMP production induced by resveratrol in bovine chromaffin cells in the absence (A) and presence (B) of IBMX. Cultured chromaffin cells were preincubated with Tyrode's solution with or without 0.5 mM IBMX at 37°C. After preincubation, they were incubated for the indicated times with resveratrol. cGMP in the cells was assayed as described under *Materials and Methods*. Experiments were performed in triplicate, and data are expressed in picomoles per 10^6 cells as mean ± S.E. from five different cellular preparations. Level of statistical significance: *, $p < 0.05$ versus control, as determined by analysis of variance/Dunnett's test.

Cardiovascular protection has been linked to the L-arginine-NO synthase signaling pathway; this is so because resveratrol augments the activities of soluble and particulate guanylate cyclase, thereby increasing cGMP levels in vascular tissues (Orallo et al., 2002) and in chromaffin cells (this study). Thus, blockade by resveratrol of quantal release could be mediated by cGMP, although the role of this nucleotide in regulating catecholamine release is controversial. For instance, O'Sullivan and Burgoyne (1990) found that NO donors augmented catecholamine release. Others found inhibition of secretion (Oset-Gasque et al., 1994; Rodriguez-Pascual et al., 1996). Augmentation by resveratrol of cGMP levels in chromaffin cells (Fig. 9) and inhibition of quantal secretion fit well in the frame of the hypothesis raised by Borges' laboratory that the activation with NO donors of the NO-guanylate cyclase-cGMP pathway will block catecholamine release by changing the affinity of the intravesicular matrix for catecholamines (Machado et al., 2000). Resveratrol has been shown to augment the circulating levels of NO (Hung et al., 2000) and the expression of endothelial nitric-oxide synthase and inducible nitric-oxide synthase in mice (Das et al., 2005). On the other hand, when given in drinking water, resveratrol offered ex vivo cardioprotection through a mechanism dependent on both NO and adenosine (Bradamante et al., 2003).

The relevance of nanomolar concentrations of resveratrol in blocking the quantal catecholamine release deserves a pharmacokinetic comment. For instance, after long-term consumption of moderate amounts of red wine containing a known concentration of resveratrol, its blood levels range from 100 nM to 1 μM (Bertelli, 2006). A detailed study in humans demonstrated that absorption after oral administration of resveratrol is very high, but only trace resveratrol concentrations in plasma were detected; the bulk of resveratrol was quickly metabolized into sulfate and glucuronic acid resveratrol derivatives or hydrogenation of the aliphatic double bond (Walle et al., 2004; Wenzel and Somoza, 2005). These low circulating levels of resveratrol cast doubts on extrapolation to humans or to in vivo animal models of disease, of the collection of biological effects of resveratrol found in vitro, that most of them required resveratrol concentrations in the range of 10 to 100 μM and even greater. However, some biological activities relevant to its putative cardioprotective effects in humans, have been described to occur in the nanomolar to 1 μM range of resveratrol concentrations. This is the case for its antiplatelet activity seen at 1 μM (Bertelli et al., 1996) or protection by 1 μM resveratrol against cold preservation-warm reperfusion damage (Plin et al., 2005). In this context, our present study shows unequivocally that 30 to 300 nM resveratrol causes an efficient and potent blockade of the quantal catecholamine release triggered by various secretagogues in chromaffin cells. This could notably contribute to the cardioprotective effects of resveratrol that have been intensely explored during the last two decades (Pervaiz,

2003; Orallo, 2006, 2008; Bertelli, 2007; Harikumar and Aggarwal, 2008; Pirola and Fröjdö, 2008).

In conclusion, we have shown that nanomolar resveratrol blocks quantal catecholamine release from single chromaffin cells stimulated with various physiological and nonphysiological secretagogues. Such an effect occurs at the very last Ca^{2+}-independent step of exocytosis and is mediated by the NO-cGMP pathway. These findings gain clinical relevance when considering that the effects of resveratrol occur at nanomolar concentrations, which can be reached in plasma after moderate consumption of red wine. They can contribute to a better understanding of the French Paradox and of the potential cardiovascular protection afforded by wine resveratrol. Mitigation by this polyphenol of the sympathoadrenal stress response could alleviate the potent arrhythmogenic effects of circulating catecholamines, which are drastically increased during stressful conflicts taking place during daily living.

Acknowledgments

We are grateful for the continued support of Fundación Teófilo Hernando, Madrid, Spain. Dr. Francisco Orallo died unexpectedly on December 9, 2009; we dedicate this work to his memory.

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