Mitochondrial calcium sequestration and protein kinase C cooperate in the regulation of cortical F-actin disassembly and secretion in bovine chromaffin cells

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Mitochondria play an important role in the homeostasis of intracellular Ca^{2+} and regulate its availability for exocytosis. Inhibitors of mitochondria Ca^{2+} uptake such as protonophore CCCP potentiate the secretory response to a depolarizing pulse of K^+. Exposure of cells to agents that directly (cytochalasin D, latrunculin B) or indirectly (PMA) disrupt cortical F-actin networks also potentiate the secretory response to high K^+. The effects of cytochalasin D and CCCP on secretion were additive whereas those of PMA and CCCP were not; this suggests different mechanisms for cytochalasin D and CCCP and a similar mechanism for PMA and CCCP. Mitochondria were the site of action of CCCP, because the potentiation of secretion by CCCP was observed even after depletion of Ca^{2+} from the endoplasmic reticulum. CCCP induced a small increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) that was not modified by the protein kinase C (PKC) inhibitor chelerythrine. Both CCCP and PMA induced cortical F-actin disassembly, an effect abolished by chelerythrine. In addition, rotenone and oligomycin A, two other mitochondrial inhibitors, also evoked cortical F-actin disassembly and potentiated secretion; again, these effects were blocked by chelerythrine. CCCP also enhanced the phosphorylation of PKC and myristoylated alanine-rich C kinase substance (MARCKS), and these were also inhibited by chelerythrine. The results suggest that the rapid sequestration of Ca^{2+} by mitochondria would protect the cell from an enhanced PKC activation and cortical F-actin disassembly, thereby limiting the magnitude of the secretory response.

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Chromaffin cells store their materials for export in membrane-bound organelles, the secretory vesicles (Trifaró & Poisner, 1982). Upon cell stimulation the vesicular content is extruded to the cell exterior by exocytosis (Trifaró & Poisner, 1982). This is a complex process of interaction between secretory vesicle components, plasma membranes and cytosolic factors leading to the fusion of vesicle and plasma membranes. Secretory vesicles are present in these cells in at least two compartments: (a) the release-ready vesicle pool and (b) the reserve pool (Heinemann *et al.* 1993; Neher & Zucker, 1993; Vitale *et al.* 1995). The traffic of vesicles between these compartments is subject to a fine regulation. Experimental evidence has demonstrated that the cortical F-actin network plays an important role in this regulation (Vitale *et al.* 1991, 1995).

Calcium ions play a pivotal role, acting at more than one level in the cascade of events leading to exocytosis. A rise in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) triggers exocytosis and this increase in Ca^{2+} is, depending of the type of stimulus, due either to Ca^{2+} entering the cell through specific channels or to Ca^{2+} being released from intracellular stores such as the endoplasmic reticulum (Kuba, 2000). Another component of the intracellular buffering machinery is the mitochondria (Duchen, 1999), which have emerged as important players in the intracellular regulation of Ca^{2+} levels (Friel & Tsien, 1994; Park *et al.* 1996; Herrington *et al.* 1996; Babcock *et al.* 1997; Montero *et al.* 2000).

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Activation of Ca^{2+} channels or Ca^{2+} release from the endoplasmic reticulum triggers fast millimolar mitochondrial Ca^{2+} transients that modulate chromaffin cell secretion (Giovannucci *et al.* 1999; Montero *et al.* 2000) as well as secretion from PC12 cells (Taylor *et al.* 2000). Exposure of chromaffin cells to protonophores abolished mitochondrial Ca^{2+} uptake and potentiated stimulated secretion; this led to the conclusion that mitochondria could regulate the availability of Ca^{2+} to the secretory machinery, and hence secretion (Montero *et al.* 2000). However, how this modulation is exerted is unknown. The purpose of the present investigation was to elucidate the mechanisms involved in the potentiation of secretion when the mitochondrial Ca^{2+} sequestration is interrupted by a protonophore. Here we demonstrate that, in chromaffin cells, the potentiation of the secretory response observed upon the collapse of the mitochondrial transmembrane electrochemical potential is accompanied by PKC and myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation and is mediated through cortical F-actin disassembly. These effects were inhibited by PKC blockers.

Methods

Materials

Phorbol 12-myristate, 13-acetate (PMA), rotenone, oligomycin A, chelerythrine chloride, and carbonyl-cyanide *m*-chlorophenyl hydrazone (CCCP) were purchased from Sigma Chemical Co. (St Louis, MO, USA); latrunculin B and cytochalasin D were obtained from Calbiochem (San Diego, CA, USA); rhodamine phalloidin and fura-2 and 1,2-bis(2-amino-phenoxy)-ethane-*N*,*N*,*N*′*N*′-tetra-acetic acid tetrakis (acetoxymethyl ester) (BAPTA AM) were purchased from Molecular Probes Inc. (Eugene, Oregon, USA). PMA, CCCP and chelerythrine were prepared in DMSO and kept as 10^{-2} M stock solutions at -20°C. Dilutions were freshly prepared on the day of the experiment, and final concentrations of DMSO were 0.1% or less in each case. Rabbit polyclonal phospho-PKC (pan) and phospho-MARCKS (ser 152/156) antibodies were obtained from Cell Signalling Technology Inc. (Beverley, MA, USA) and mouse monoclonal antibody against tubulin was purchased from Sigma Chemical Co.

Isolation and culture of chromaffin cells

Bovine adrenal glands were obtained from a local slaughterhouse. Chromaffin cells were isolated by adrenal medulla digestion with collagenase, according to the procedure of Trifaró & Lee (1980) with some modifications (Moro *et al.* 1990). Our preparations were enriched in adrenaline-containing cells. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. Cells (5×10^6 in 10 ml DMEM) were plated in 5 cm diameter Petri dishes and kept in a water-saturated incubator at 37°C, in a 5% CO_2–95% air atmosphere, and used 3–5 days thereafter. The culture medium was replaced by serum-free DMEM 24 h later, and then every 2 days.

On-line measurement of catecholamine release from bovine chromaffin cells

Cells were scraped off carefully from the bottom of the Petri dish with a rubber policeman, and centrifuged at 800 *g* for 10 min. The cell sediment was resuspended in 200 μl of Krebs-Hepes (composition (mM): NaCl 144; KCl 5.9; $MgCl_2$ 1.2; glucose 11; Hepes 10; pH 7.4); the concentration of $CaCl_2$ varied depending on the protocol. Cells were introduced to a jacketed microchamber for their superfusion at 37°C. The superfusion rate was 2 ml min^{-1}. The liquid flowing from the superfusion chamber reached an electrochemical detector (model Metrohn AG CH-9100 Hersau) placed just at the outlet of the microchamber that monitored, 'on line' under the amperometric mode, the amount of catecholamines secreted (Borges *et al.* 1986). Cells were stimulated to secrete with short pulses (1–5 s) of high K^+ Krebs-Hepes solution controlled by electro-valves. Depolarizing concentrations of K^+ were applied in the presence or absence of different test compounds when cells were being superfused with Krebs-Hepes solution (see Results for further details).

Measurement of changes of $[Ca^{2+}]_c$ in fura-2-loaded bovine chromaffin cells

Chromaffin cells were loaded with fura-2 by incubating them with fura-2 AM (4 μM) for 60 min at 37°C in Krebs-Hepes solution (pH 7.4) containing (mM): NaCl 144; KCl 5.9; $MgCl_2$ 1.2; $CaCl_2$ 2.4; sodium Hepes 10; glucose 10. The loading incubation was terminated by washing the coverslip containing the attached cells several times with Krebs-Hepes solution. The coverslip containing the fura-2-loaded cells was placed in a chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature ($22 \pm 2^{\circ}$C) with Krebs-Hepes solution. Once selected, an individual cell was locally superfused with various solutions that were changed 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 being explored. The flow rate was smaller than 1 ml min^{-1} and was regulated by gravity to achieve complete replacement of the cell surroundings within less than 1 s. This allowed

the precise application of depolarizing pulses of K^+ of 5 s duration.

The fluorescence of fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of the $[Ca^{2+}]_c$. Fura-2 was excited with light alternating between 360 and 390 nm, using a Nikon 40 × fluorite objective. Emitted light was transmitted through a 425 nm dichroic mirror and a 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_c$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz *et al.* 1985).

Protein phosphorylation

A PKC phosphorylation assay was performed according to the manufacturer's instructions (Cell Signalling Technology Inc.). Briefly, 1×10^6 chromaffin cells were incubated with Krebs-Hepes buffer in the absence (control) or presence of PKC inhibitors and then 2 μM CCCP in Krebs-Hepes buffer was added for 90 s. When PKC inhibitors were used, cells were pre-exposed to 1 μM of RO31-8220, staurosporine or chelerythrine for 8.5 min and then incubated with 2 μM CCCP for 90 s. The reaction was stopped by addition of 1 × SDS Sample Buffer (62.5 mM Tris, pH 6.8, 2% w/v SDS, 20% glycerol, 50 mM DTT, 0.5 mM EDTA, 0.5 mM EGTA), supplemented with the protease inhibitors phenymethylsulphonyl fluoride (0.5 mM), leupeptin (10 μg ml^{-1}) and aprotinin (10 μg ml^{-1}), as well as phosphatase inhibitors sodium fluoride (1 mM) and sodium orthovanadate (0.1 mM), followed by boiling for 5 min. Samples, each containing an equal amount of protein, were separated by SDS-PAGE and then electrotransferred onto a nitrocellulose membrane for analysis of PKC phosphorylation by Western blotting. Primary antibodies were rabbit polyclonal phospho-PKC (pan) antibody (1 : 500), phospho-MARCKS (ser 152/156) rabbit polyclonal antibody (1 : 500) and mouse monoclonal anti-tubulin antibody (1 : 2000). Following incubation with the corresponding goat (anti-rabbit or anti-mouse) horseradish peroxidase conjugated IgG (1 : 3000) (Bio-Rad Laboratories, Hercules, CA, USA), the membranes were incubated in electrochemiluminescence (ECL) Western blotting detection reagents (Amersham, Oakville, Canada). Chemiluminescence-emitting signals were detected by Hyperfilm ECL. Multiple exposures of each set of samples were carried out and autoradiographs were scanned. The density and area of the bands were calculated with the Scion Image Beta-3b software (Scion Corporation, Frederick, MD, USA). Values obtained for each phospho-PKC band were normalized on the basis of the tubulin band intensity (loading control) to correct for minor variations in loading.

Fluorescence microscopy

Chromaffin cells were plated on collagen-coated coverslips contained within plastic Petri dishes at a density of 3×10^5 cells per 35 mm dish. Cells were rinsed with Krebs-Hepes solution. Cells were incubated for different periods of time with Krebs-Hepes solution and in the absence or presence of different compounds; then they were stimulated by high K^+, fixed in 3.7% formaldehyde and processed for fluorescence microscopy (Lee & Trifaró, 1981). Chromaffin cells were stained with rhodamine phalloidin (a probe for filamentous actin; 1.25 U ml^{-1}) and only changes in the cortical actin network of chromaffin cells were recorded and analysed. Coverslips were thoroughly washed with phosphate-buffered saline (PBS). Finally coverslips were rinsed with PBS and mounted in glycerol–PBS (1 : 1). Slides were viewed with a Leitz Ortholux fluorescence microscope and photographs were taken with Kodak-Tri-X pan films (400 ASA) (Vitale *et al.* 1991). To study the effect of several treatments on cortical F-actin disassembly, 100 single rounded chromaffin cells per coverslip (usually 6–8 coverslips per experimental condition) were examined. Each cell was classified as having either a 'continuous' or 'discontinuous' cortical rhodamine (F-actin) fluorescent ring (Vitale *et al.* 1991). The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition. 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. The codes were revealed to identify the experimental conditions used only after all results were recorded (single-blind design).

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 set up to obtain a clear image of the cell on the monitor and a fluorescence intensity of 250 (arbitrary units) in the cortical region of the cell. 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).

Data analyses and statistics

Data are presented as means ± standard errors of the mean (S.E.M.) and were analysed by one-way ANOVA. Scheffe's

test was used to determine the level of significance of differences between groups. Results shown in Figs 4 and 6 were analysed by Student's *t* test.

Results

Effect of collapsing mitochondrial transmembrane H^+ gradient on secretion

Recent published work has demonstrated that short exposure (90 s) of chromaffin cells to 2 μM of the protonophore cyanide *m*-chlorophenyl hydrazone (CCCP) potentiated the secretory response to a short depolarizing pulse of K^+ (Montero *et al.* 2000). The strong potentiating effect on K^+ depolarization-evoked amine release observed with CCCP treatment was similar to that detected when cells were exposed to 100 nM phorbol ester (PMA; Fig. 1*A* and *D*). It is known that potentiation of the chromaffin cell secretory response by PMA is mainly due to a disruption of the cortical F-actin networks allowing the movements of chromaffin vesicles to release sites on the plasma membrane (Vitale *et al.* 1995). Another way to disrupt cortical F-actin networks is through direct interactions with actin filaments, as was the case for latrunculin B or cytochalasin D treatments (Fig. 1*B–D*). However, in this situation, the potentiation of high K^+-evoked amine release was smaller than that observed with either CCCP or PMA.

When cytochalasin D and CCCP were applied together, the potentiation of the high K^+-evoked response was even larger, showing summation of effects (Fig. 2*A* and *C*). This would suggest that two different mechanisms might be involved in the potentiation of the secretory response to depolarization. On the other hand, there was no summation of effects during simultaneous perfusion with CCCP and PMA, although the secretory response to high K^+ was a little bit larger than that obtained with either one of the compounds (Fig. 2*B* and *C*). It seems therefore possible that the same transduction pathway might be involved in the response to treatment with either

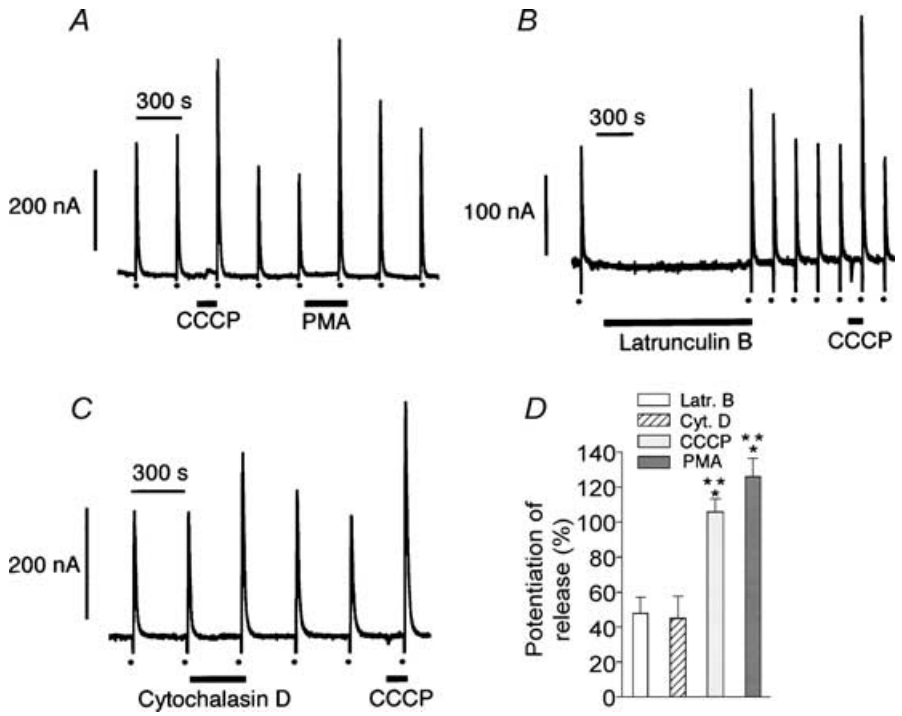

Figure 1. CCCP, PMA, latrunculin B and cytochalasin D enhance secretory responses induced by depolarizing concentrations of K^+

Chromaffin cells were superfused with Krebs-Hepes medium at 37°C. After a 10 min equilibration period, cells were stimulated at 5 min intervals with 5 s pulses of medium containing high K^+ (150 mM with isosmotic reduction of Na^+). High K^+ challenges are represented by dots at the bottom of the panels. *A*, in this and the following experiments, 2 μM CCCP was applied 90 s prior to the high K^+ pulse. PMA (100 nM) was applied 5 min prior to the depolarizing K^+ pulse. Latrunculin B (5 μM) and cytochalasin D (2 μM) were applied 20 (*B*) and 5 (*C*) min, respectively, prior to the high K^+ pulse. Amplitudes of secretion peaks are expressed as oxidation currents in nA. *D*, potentiation of high K^+-evoked amine release by latrunculin B ($n = 11$), cytochalasin D ($n = 6$), CCCP ($n = 34$) and PMA ($n = 10$). Bars represent means ± S.E.M. $^*P < 0.01$ *versus* latrunculin B; $^{**}P < 0.01$ *versus* cytochalasin D.

CCCP or PMA. When cells were perfused with 100 nM PMA followed by 2 μM CCCP, the secretory response to high K^+ was only potentiated during perfusion with PMA; no potentiation was observed in the presence of CCCP (Fig. 3*A* and *C*), suggesting, again, that the same transduction pathway was utilized in the potentiation of amine release produced by either of the two compounds. It is known that the effects of PMA on chromaffin cells are mediated through activation of protein kinase C (PKC) (Vitale *et al.* 1992, 1995). In the present experiments, 1 μM chelerythrine (an inhibitor of PKC), when present prior to and during perfusion with 2 μM CCCP, abolished the potentiating effects of CCCP on high K^+ responses (Fig. 3*B* and *C*).

Changes of the $[Ca^{2+}]_c$ evoked by CCCP

These experiments were performed in fura-2-loaded, single chromaffin cells, at room temperature. Figure 4*A* shows the original $[Ca^{2+}]_c$ traces obtained in an example cell. After the $[Ca^{2+}]_c$ reached a stable baseline (at about 100 nM concentration) a K^+ pulse (70 mM K^+, 5 s duration) caused a sharp rise of $[Ca^{2+}]_c$ that declined quickly to reach basal levels in about 1 min. The cell was subsequently superfused with cytochalasin D (2 μM), which did not change the basal $[Ca^{2+}]_c$. Then a second pulse of K^+ was added, followed by the application of CCCP (2 μM), which produced a slow rise of $[Ca^{2+}]_c$ that reached a plateau at around 200 nM and returned quickly to the basal level upon washing out CCCP.

Similar experiments were performed in other cells to test the effect of PMA. For instance, Fig. 4*B* shows a cell that was first challenged with K^+ and subsequently with CCCP. Note that, again, CCCP caused a mild and sustained elevation of the $[Ca^{2+}]_c$; note also that PMA (100 nM) did not change the basal levels of $[Ca^{2+}]_c$. An example of a third protocol is shown in Fig. 4*C*. Note that CCCP produced its typical $[Ca^{2+}]_c$ elevation effect. When the cell was superfused with chelerythrine (100 μM), there was no change in the basal levels of Ca^{2+}. When applied in the presence of chelerythrine, CCCP produced an elevation of the $[Ca^{2+}]_c$ that was slightly higher. However, averaged results of four experiments show no changes in the kinetic parameters observed for the CCCP-evoked changes in $[Ca^{2+}]_c$, i.e. peak amplitude, time to peak and area of the Ca^{2+} curve, which represents the total Ca^{2+} accumulated in the cytosol during the CCCP challenge. Note that chelerythrine did not modify either the basal $[Ca^{2+}]_c$ or the kinetic parameters of the $[Ca^{2+}]_c$ elevation elicited by CCCP.

Effect of CCCP on chromaffin cell cortical F-actin networks

The chromaffin cell possesses a cortical network of filamentous actin underneath the plasma membrane which is disrupted by cholinergic receptor stimulation, cell depolarization or PMA treatment (Vitale *et al.* 1991, 1995). Therefore, one of the effects of PKC activation responsible for the potentiation of the secretory

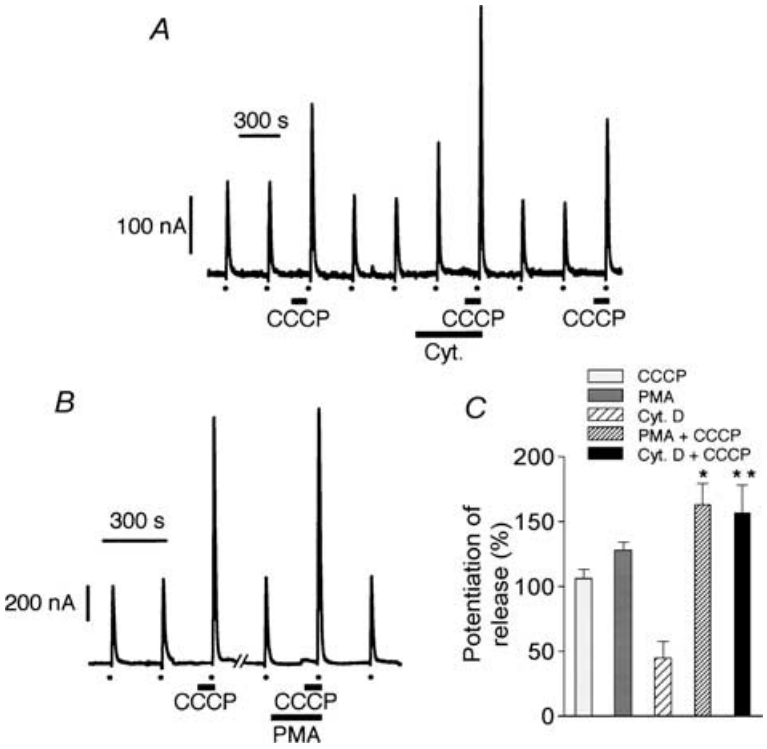

Figure 2. Effects of cytochalasin D and PMA on the potentiation of K^+-evoked amine release produced by CCCP
A, secretion peaks in nA obtained during 5 s high K^+ (150 mM) pulses (dots at the bottom of panels) in the absence or presence (90 s prior to stimulation) of CCCP. Cytochalasin D (2 μM) was present in the medium 5 min prior to and during the second 90 s perfusion period with CCCP. *B*, similarly, 100 nM PMA was present in the medium 3.5 min prior to and during the 90 s perfusion with CCCP. *C*, potentiation of high K^+-induced amine release by CCCP ($n = 29$), PMA ($n = 6$), cytochalasin D ($n = 6$), PMA + CCCP ($n = 8$), and cytochalasin D + CCCP ($n = 8$). Bars represent mean ± S.E.M. $^*P < 0.01$ *versus* CCCP or PMA; $^{**}P < 0.01$ *versus* cytochalasin D. Other conditions were as described in legend to Fig. 1.

response is the disassembly of cortical filamentous-actin networks (Vitale *et al.* 1995), an effect mediated through the phosphorylation of MARCKS (Rosé *et al.* 2001). Treatment of chromaffin cells for 90 s with 2 μM CCCP produced cortical F-actin disassembly as evaluated by fluorescence microscopy of rhodamine–phalloidin (a probe for filamentous actin) stained cells (Fig. 5*A* and *B*). The fluorescence images of cortical F-actin disassembly obtained were quite similar to those observed after 100 nM PMA or high K^+ treatment of chromaffin cells (Vitale *et al.* 1995). Moreover, the disruption in the cortical F-actin fluorescence ring produced by CCCP was inhibited by treatment with 1 μM chelerythrine (Fig. 5*A* and *B*), suggesting the involvement of PKC in this process. Similarly to PKC stimulation, CCCP-induced cortical F-actin disassembly was not dependent on extracellular Ca^{2+}. CCCP-induced F-actin disassembly was observed in $76.9 \pm 3.2\%$ ($n = 400$) of the cells in the presence of extracellular Ca^{2+}, and in $74.1 \pm 1.8\%$ ($n = 600$) of the cells in Ca^{2+}-free medium. Moreover, F-actin disassembly in Ca^{2+}-free medium in response to CCCP was inhibited by $98.8 \pm 6.6\%$ ($n = 600$) by preincubation of cells for 30 min with 50 μM BAPTA AM.

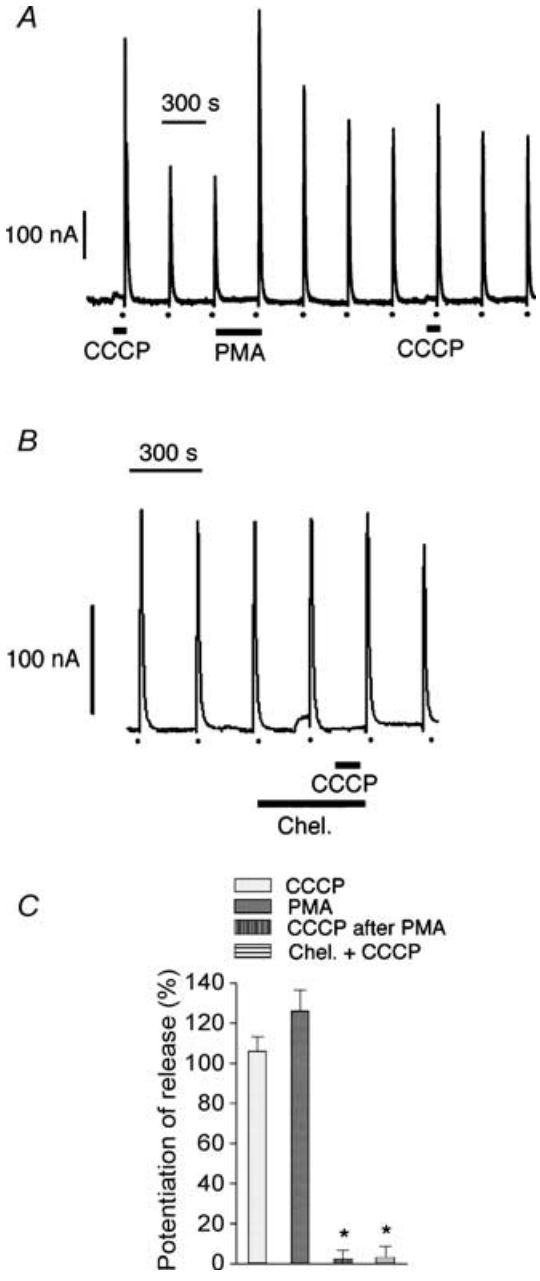

Figure 3. Effect of PMA and chelerythrine on the potentiation of K^+-induced amine release produced by CCCP
Secretion peaks in nA obtained during 5 s pulses of depolarizing concentrations (150 mM) of K^+ (dots at the bottom of the panels). *A*, PMA (100 nM) and CCCP (2 μM) were perfused 5 min and 90 s, respectively, prior to the high K^+ challenges. *B*, chelerythrine (1 μM) was present in the medium 8.5 min prior to and during the 90 s period of perfusion with CCCP. *C*, potentiation of high K^+-evoked amine release by CCCP ($n = 36$), PMA ($n = 6$), CCCP after PMA ($n = 5$) and CCCP in the presence of chelerythrine ($n = 6$). Bars represent mean ± S.E.M. $^*P < 0.01$ *versus* CCCP. Other conditions were as described in legend to Fig. 1.

Correlation between cortical F-actin disassembly and catecholamine output and their concentration-dependent inhibition by PKC inhibitors

Potentiating by CCCP of high K^+ -evoked catecholamine output and cortical F-actin disassembly was measured in the presence or absence of increasing concentrations of chelerythrine, staurosporine or RO31-8220 (Fig. 6*A*–*C*), three PKC inhibitors. The three blockers produced a concentration-dependent inhibition of both responses, suggesting the involvement of PKC in CCCP potentiation of these two high K^+-evoked effects. Moreover each blocker produced the same degree of inhibition for both catecholamine release and F-actin disassembly (Fig. 6*A*–*C*). Thus, as expected each inhibitor showed similar IC_{50} values for catecholamine output and F-actin disassembly. IC_{50} values were (μM): chelerythrine, 0.35 and 0.46, staurosporine, 0.22 and 0.20, RO31-8220, 0.013 and 0.016 for catecholamine output and F-actin disassembly, respectively.

Effect of CCCP on PKC and MARCKS phosphorylation

Phosphorylation of PKC is an indication of its activation (Keranen *et al.* 1995). Therefore, the effect of 90 s CCCP exposure on chromaffin cell PKC phosphorylation was measured by Western blotting using an antibody specific for phosphorylated PKC. Under these experimental conditions, a 50% increase in PKC phosphorylation was observed (Fig. 6*D* and *E*). As expected, CCCP-evoked PKC phosphorylation was inhibited by the three PKC blockers.

Furthermore, experiments from our laboratory have shown that cortical F-actin disassembly induced by PKC activation is mediated by phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS)

(Rosé *et al.* 2001), a PKC substrate. Unphosphorylated MARCKS cross-links actin filaments whereas phosphorylated MARCKS decreases F-actin cross-linking and promotes F-actin disassembly (Hartwig *et al.* 1992; Rosé *et al.* 2001). An increase in MARCKS phosphorylation was observed 90 s following CCCP exposure (Fig. 6*F* and *G*) and, again, this effect was abolished by PKC inhibitors. MARCKS phosphorylation was similar in the presence ($70 \pm 9.5\%$ increase in phosphorylation, $n = 8$) or absence ($61 \pm 8.2\%$, $n = 6$) of extracellular Ca^{2+}. Moreover, the increase in MARCKS phosphorylation produced by CCCP in Ca^{2+}-free medium was inhibited $100 \pm 8\%$ ($n = 6$) by 30 min preincubation with 50 μM BAPTA AM.

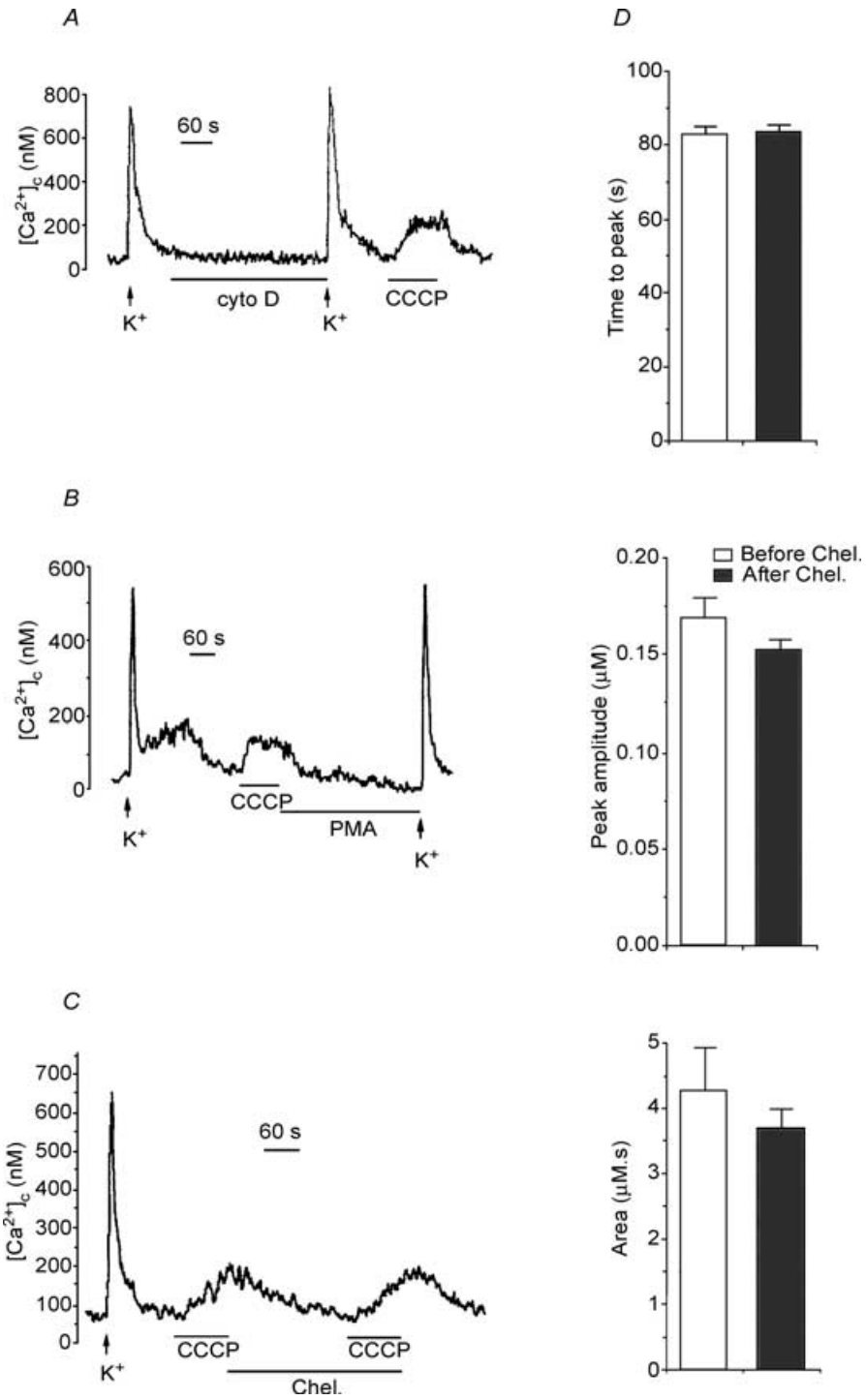

Figure 4. Ca^{2+} transients during exposure to high K^+ or CCCP
Chromaffin cells cultured on coverslips were loaded with fura-2 and $[Ca^{2+}]_i$ was measured at a single cell level as described in Methods. Two 5 s pulses of high K^+ (70 mM K^+, lower Na^+ solution) (arrows) were given to each cell, at the beginning and end of each experiment. Cytochalasin D (2 μM), CCCP (2 μM), PMA (100 nM) and chelerythrine (1 μM) were applied during the time indicated by the horizontal bars in *A*–*C*. *D*, comparison of kinetic parameters of $[Ca^{2+}]_i$ transients evoked by CCCP before and after chelerythrine exposure. The top histogram shows the time to peak in seconds, the middle histogram shows the amplitude of the plateau of $[Ca^{2+}]_c$, in the presence of CCCP, and the lower histogram shows the area of the $[Ca^{2+}]_c$ curve, expressed in μM s. Bars represent mean ± S.E.M. of 4 experiments.

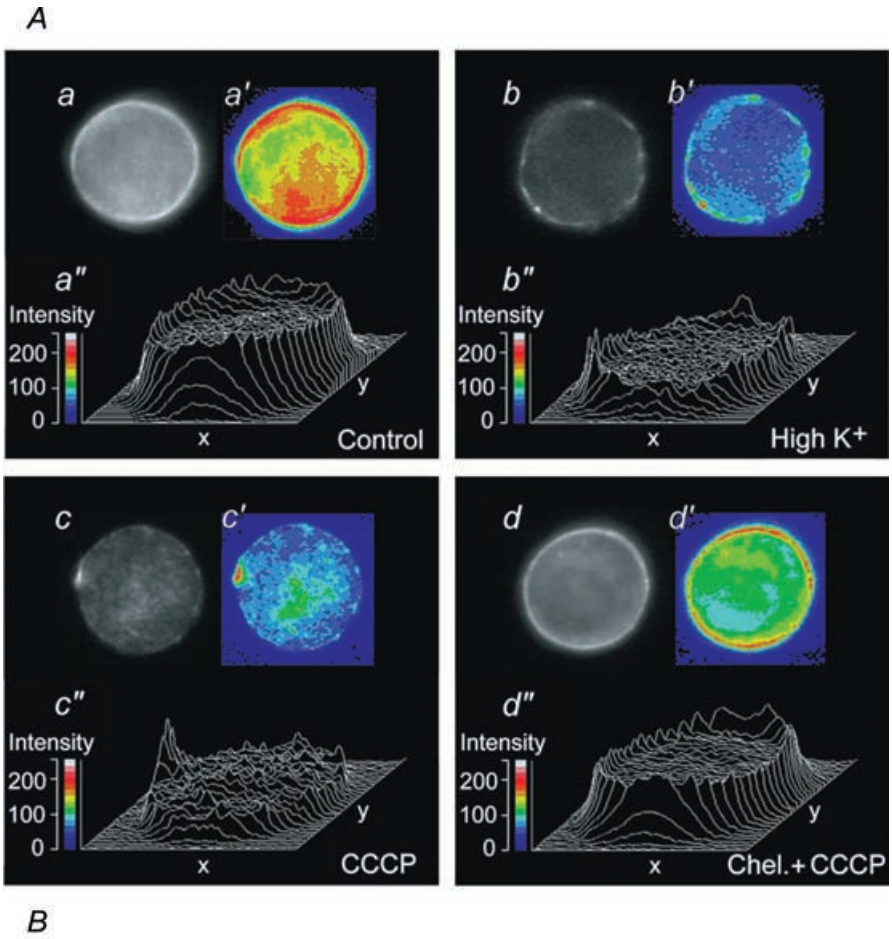

Figure 5. Effect of CCCP on chromaffin cell cortical F-actin networks

Cells cultured on coverslips were stimulated by either a 5 s pulse of high K^+ or a 90 s exposure to 2 μM CCCP. In some experiments, 1 μM chelerythrine was present 8.5 min prior to and during the 90 s exposure to CCCP. *A*, fluorescence microscopy and video-enhanced image analysis of F-actin fluorescent profiles of a single control (*a*, a′, a″) cell or cells stimulated with high K^+ (*b*, *b*′, *b*″), CCCP (*c*, *c*′, *c*″) or CCCP in the presence of chelerythrine (*d*, *d*′, *d*″). Image *a* shows a resting chromaffin cell after rhodamine–phalloidin treatment. A weak cytoplasmic staining and a continuous and bright cortical fluorescent ring is observed. Stimulation of cells with either high K^+ (*b*) or CCCP (*c*) caused a disruption of the cortical fluorescent ring. In the presence of chelerythrine, CCCP was unable to induce disruption of the cortical fluorescent ring (*d*). Images *a*′, *b*′, *c*′ and *d*′ and three-dimensional plots of fluorescence intensities (a″, *b*″, *c*″ and *d*″) of the same cells shown in a′, *b*′, *c*′ and *d*′. Fluorescence intensities of a′, *b*′, *c*′ and *d*′ are depicted in pseudo-colours according with the scales shown in arbitrary units. *B*, under the fluorescence microscope the rhodamine cortical staining was analysed and classified as being 'continuous', as in *Aa* and *a*′ and *Ad* and *d*′, or 'discontinuous', as in *Ab* and *b*′ and *Ac* and *c*′, and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) in control and treated preparations was calculated as indicated in Methods. For each experimental condition, 400 cells from at least 3 different cultures were examined. Bars represent mean ± S.E.M. $^*P < 0.01$ *versus* control; $^{**}P < 0.01$ *versus* CCCP.

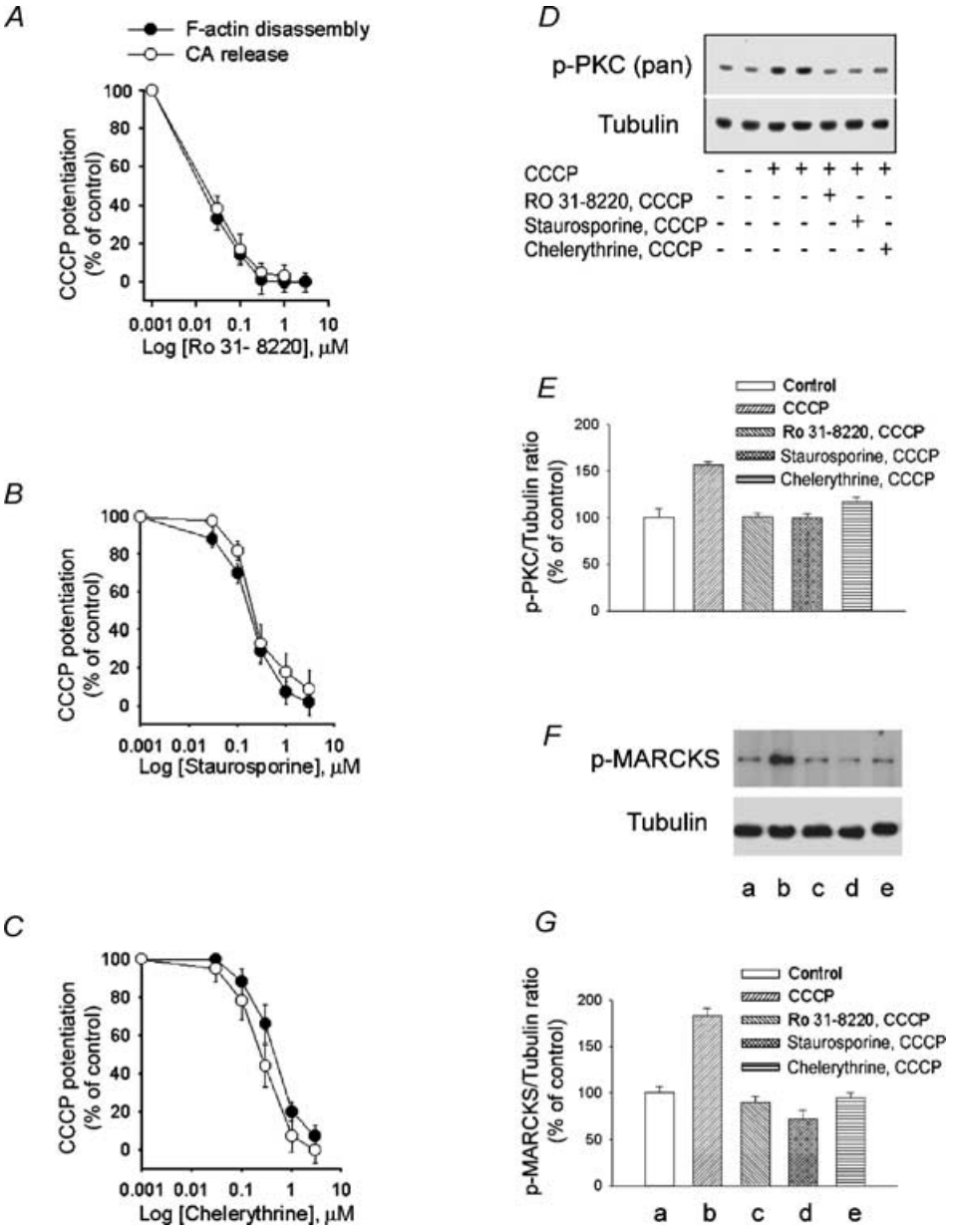

Figure 6. Effects of PCK inhibitors on the potentiation by CCCP of cortical F-actin disassembly, catecholamine secretion and PKC and MARCKS phosphorylation

A–C, CCCP potentiation of high K^+ depolarization-induced F-actin disassembly and catecholamine release was inhibited by RO31-8220, staurosporine and chelerythrine in a concentration-dependent manner. *D*, Western blot analysis of extracts from CCCP-treated bovine chromaffin cells in the presence or absence of PKC inhibitors. Chromaffin cells were pre-exposed to a Krebs-Hepes solution (control) or to the same solution containing either 1 μM of each of the PKC inhibitors RO31-8220, staurosporine or chelerythrine during 8.5 min. This was followed by incubation with Hepes solution (control) or 2 μM CCCP in Hepes solution for 90 s. The reaction was stopped by addition of 'Sample buffer' as described in Methods. Proteins in whole cell lysates were detected by Western blotting with phospho-PKC (pan), and tubulin antibodies. Tubulin was used as the gel-loading control. *E,* PKC phosphorylation expressed as percentage increase in phospho-PKC content relative to unstimulated (control) cells. Bars represent mean ± S.E.M. of data obtained from 3 different experiments. $^*P < 0.01$ *versus* control. *F,* CCCP-induced phosphorylation of MARCKS. Chromaffin cells were incubated for 90 s with either Krebs-Hepes solution or the solution containing 2 μM CCCP. Cell extracts were prepared and proteins were separated by SDS-PAGE followed by Western blotting with phospho-MARCKS and tubulin antibodies. Tubulin was used as the gel-loading control. The effect of PKC inhibitors on CCCP-induced MARCKS phosphorylation is also shown. *G,* MARCKS phosphorylation expressed as percentage increase in phospho-MARCKS content relative to un-stimulated (control) cells. Letters a to e below the bars correspond to the equivalently labelled lanes in *F*. Bars represent mean ± S.E.M. of data obtained from 8 different experiments. $^*P < 0.01$ *versus* control.

Specificity of CCCP effects: Is mitochondria the only target for CCCP?

It has been suggested that CCCP may have other intracellular targets such as the endoplasmic reticulum (ER) and therefore the possibility exists that the effects observed here with CCCP were the result of Ca^{2+} being released from the ER. To test this possibility, the ER Ca^{2+} store was depleted by 30 min treatment with a combination of 10 mM caffeine, 10 μM ryanodine and 1 μM thapsigargin. This treatment releases ER Ca^{2+}, leaves the ryanodine receptor channel of the ER in a low conductance open state (Alonso *et al.* 1999) and, in addition, blocks Ca^{2+} uptake into the ER. Under these conditions, the CCCP potentiating effect on high K^+-evoked secretory responses was maintained (Fig. 7) whereas the secretory response to caffeine was abolished (data not shown). Furthermore, when mitochondrial respiration and ATP production were blocked by treatment with a combination of 4 μM rotenone and 3 μM oligomycin A, a potentiation of the high K^+-evoked secretion was also observed (Fig. 8*A* and *D*). As with the CCCP-evoked potentiation of depolarization-evoked amine release, pretreatment with 100 nM PMA significantly reduced the rotenone and oligomycin A-induced potentiation (Fig. 8*B* and *D*) and, in addition, 1 μM chelerythrine produced a complete blockade (Fig. 8*C* and *D*), suggesting the involvement of PKC in the process. Cortical F-actin cytoskeleton disassembly was also observed with this combined treatment (Fig. 8*E*). The fluorescence images of cortical F-actin disassembly obtained following rotenone–oligomycin A treatment were quite similar to those observed upon CCCP treatment. Moreover, the effects of rotenone–oligomycin A on the cytoskeleton were blocked by 1 μM chelerythrine (Fig. 8*E*).

Discussion

Several publications (Giovannucci *et al.* 1999; Montero *et al.* 2000; Taylor *et al.* 2000), including the present work, have shown that treatment with mitochondrial inhibitors, such as the protonophore CCCP, potentiates the secretory response to chromaffin and PC12 cell stimulation. In the present experiments, the exposure of chromaffin cells to 2 μM CCCP was for 90 s prior to the depolarizing pulse of K^+ (3.5 min after the previous stimulus). Under these conditions, the secretory response was potentiated 2–2.5 times (Fig. 1). It seems that during the exposure to CCCP, enough Ca^{2+} was accumulated to potentiate the secretory response to high K^+. Indeed, exposure of chromaffin cells to 2 μM CCCP for 90 s increased cytosolic Ca^{2+} even when total cytosolic Ca^{2+} had apparently reached resting levels (Fig. 4*C*). This would suggest that, under resting conditions, there would be cellular microenvironments where the level of intracellular Ca^{2+} would be in the range of at least 2–3 μM (Montero *et al.* 2000), a concentration which is enough to evoke uptake into mitochondria and, consequently, observe the effect of CCCP.

As demonstrated here, other treatments such as exposure to cytochalasin D, latrunculin B or phorbol esters (PMA) also potentiated the secretory response to a depolarizing pulse of K^+. However, exposure to these

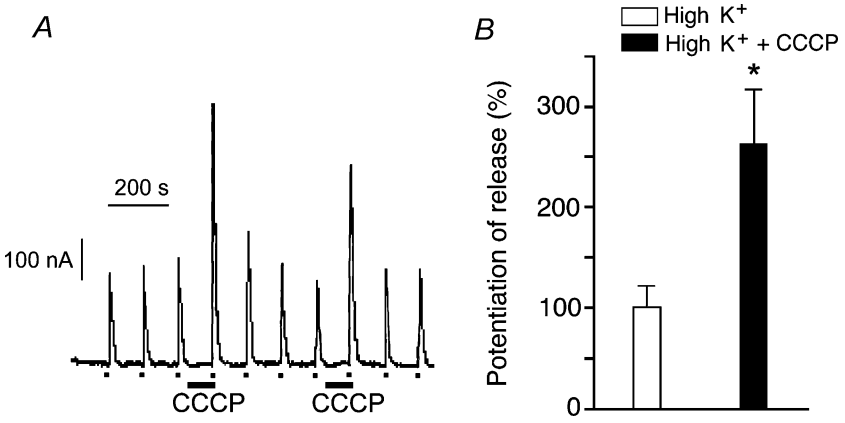

Figure 7. The potentiating effect of CCCP on high K^+-evoked amine release remains after depletion of endoplasmic reticulum (ER) Ca^{2+} stores
Chromaffin cell ER Ca^{2+} stores were depleted by 30 min treatment with a combination of 10 mM caffeine, 10 μM ryanodine and 1 μM thapsigargin in Ca^{2+}-free medium as previously described (Alonso *et al.* 1999). Cells were then superfused with regular Krebs-Hepes solution and stimulated with 5 s pulses of high K^+ (dots at the bottom of the panel). *A*, when cells were perfused with 2 μM CCCP for 90 s prior to the high K^+ pulse, the secretory response to high K^+ was significantly potentiated. *B*, bars represent mean ± S.E.M. ($n = 11$) of amine secretion during high K^+ pulses (control; the secretory response to high K^+ was considered to be 100%) or the pulses preceded by 90 s exposure to 2 μM CCCP. Secretion was calculated by integration of the areas of the secretory peaks and then expressed as percentages. *$P < 0.01$ *versus* high K^+.

substances did not modify resting intracellular Ca^{2+} levels (Fig. 4). It is known that PMA potentiates chromaffin cell responses to different secretagogues (Burgoyne & Norman, 1984; Pocotte *et al.* 1985; Brocklehurst *et al.* 1985; Burgoyne *et al.* 1988; Knight *et al.* 1988; Terbush *et al.* 1988; Bittner & Holz, 1990; Tachikawa *et al.* 1990; Vitale *et al.* 1992, 1995; Smith *et al.* 1998; Rosé *et al.* 2001). It is also known that PMA treatment does not induce secretion but disrupts chromaffin cell cortical F-actin networks and increases both the number of secretory vesicles at the subplasmalemmal zone (release-ready vesicle pool) and the initial rate of exocytosis in response to stimulation (Vitale *et al.* 1995). Indeed, membrane capacitance studies showed, in PMA-treated cells, an increased number of vesicles fusing with the plasma membrane during cell depolarization (Vitale *et al.* 1995; Smith *et al.* 1998). The chromaffin cell cortical F-actin network acts as a barrier to the secretory vesicles by blocking their movement toward the plasma membrane (Trifaró *et al.* 1982, 1985; Cheek & Burgoyne, 1986; Burgoyne & Cheek, 1987; Sontag *et al.* 1988; Burgoyne *et al.* 1989). Cell stimulation is accompanied by a focal transient disassembly of the cortical F-actin network (Cheek & Burgoyne, 1986; Vitale *et al.* 1991). This allows the movement of additional vesicles from the reserve pool to release sites at the plasma membrane (Vitale *et al.* 1995).

Cytochalasin D is a fungal toxin that depolymerizes F-actin and disrupts actin filaments (Brenner & Korn, 1979; Flanagan & Lin, 1980; Schliwa, 1982) and latrunculin B is a unique marine sponge toxin that also disrupts actin microfilaments, but in this case, by blocking actin polymerization (Spector *et al.* 1983). These two toxins did not cause secretion on their own but, as demonstrated here, they potentiated the secretory response. It is therefore quite possible that the potentiating effect of the toxins was due to the movement of secretory vesicles to the subplasmalemma area prior to the depolarizing stimulus; this being the result of the disruption of cortical F-actin (Gil *et al.* 2000). Perfusion of chromaffin cells with a combination of

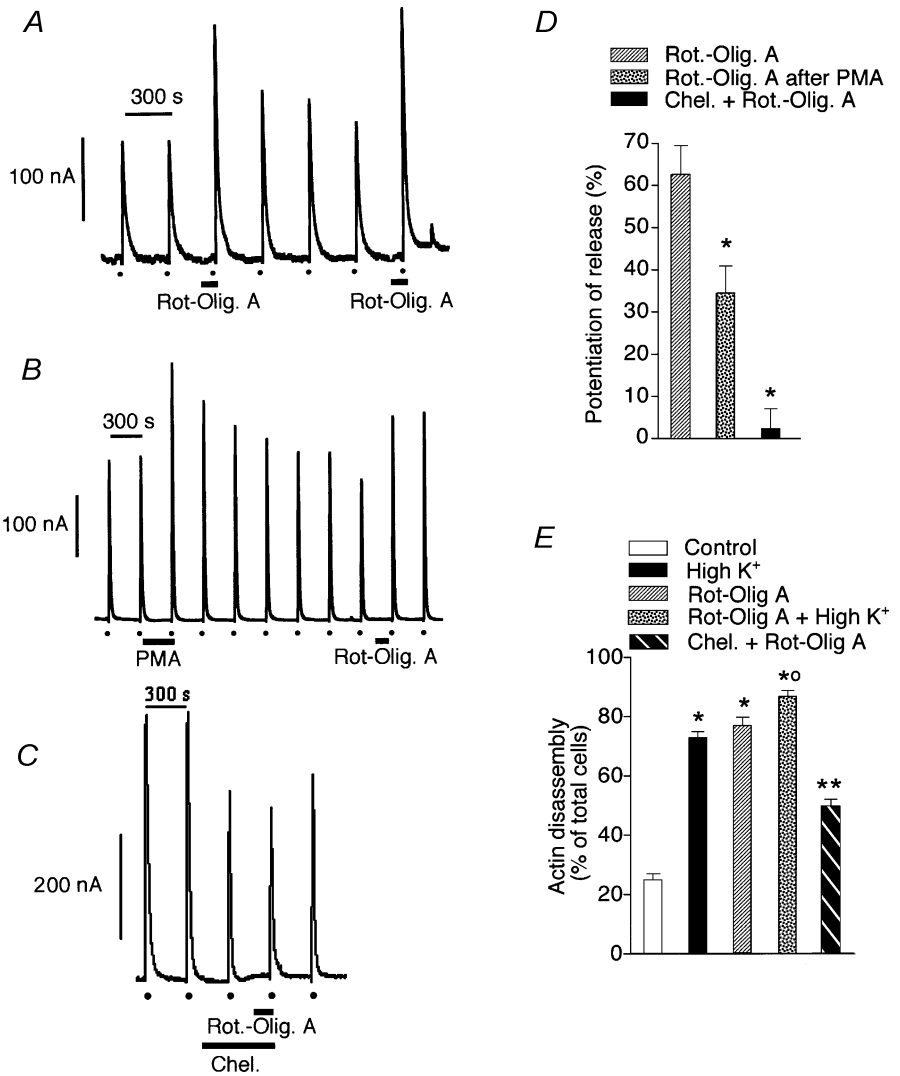

Figure 8. Rotenone and oligomycin A potentiate high K^+-evoked amine release
A–C, amine secretion peaks in nA obtained during 5 s pulses of depolarizing K^+ (dots at the bottom of panels). A combination of 4 μM rotenone and 3 μM oligomycin A was superfused, when indicated by horizontal bars, prior to the high K^+ pulse. Chelerythrine (1 μM) was superfused 8.5 min prior to and during the rotenone–oligomycin A exposure. *D*, potentiation of high K^+-induced amine release by rotenone–oligomycin A ($n = 7$), rotenone–oligomycin A following PMA exposure ($n = 5$), and rotenone–oligomycin A in the presence of chelerythrine ($n = 6$). Bars represent mean ± S.E.M. $^*P < 0.01$ *versus* rotenone–oligomycin A. *E*, chromaffin cell cortical F-actin disassembly in control ($n = 5$) and in cells stimulated for 5 s with high K^+ ($n = 4$), 90 s exposure to rotenone–oligomycin A in the absence ($n = 5$) or presence of high K^+ ($n = 4$) during the last 5 s exposure to the compounds, and rotenone–oligomycin A in the presence of 1 μM chelerythrine ($n = 4$). The rhodamine cortical staining was analysed as described in the legend to Fig. 5. Bars represent the mean ± S.E.M. $^*P < 0.01$ *versus* control; $^{**}P < 0.01$ *versus* rotenone–oligomycin A; †$P < 0.01$ *versus* high K^+ or rotenone–oligomycin A.

cytochalasin D and CCCP produced a summation of effects in the potentiation of high K^+ secretory responses (Fig. 2), suggesting the involvement of different mechanisms. On the other hand, the combination of PMA and CCCP did not show summation of effects, although responses to stimulation were larger than those in the presence of either substance (Fig. 2). This observation suggested to us that PMA and CCCP elicited potentiation of the secretory response through the same final common pathway. Indeed, when the CCCP pulse was preceded by a PMA pulse the potentiating effect of CCCP was completely abolished (Fig. 3), suggesting, again, a common pathway for these effects. The effect of PMA on secretion is the result of PKC stimulation (Vitale *et al.* 1992, 1995) since the effect is blocked by PKC inhibitors (Vitale *et al.* 1992). Chelerythrine, staurosporine and RO31-8220, three known inhibitors of PKC, also blocked the potentiating effect of CCCP (Fig. 6), suggesting the involvement of PKC in the effects elicited by this compound. Indeed, exposure to CCCP for 90 s increased PKC and MARCKS phosphorylation, effects blocked by the three PKC inhibitors. Published work from our laboratory has also demonstrated that MARCKS, a PKC substrate, mediates, at least in part, the effect of PKC on secretion (Rosé *et al.* 2001). In view of these observations, immunocytochemical studies with rhodamine-labelled phalloidin, a probe for filamentous actin, indicated, as expected, that exposure of chromaffin cells to CCCP induced cortical F-actin disassembly which was inhibited by the three PKC blockers (Fig. 6), suggesting, again, the involvement of PKC in this process. Cortical F-actin disassembly was also observed during the exposure of resting cells to CCCP. Under these conditions, no increase in secretion was observed. These results are similar to those obtained with PMA, a substance which evoked cortical F-actin disassembly without inducing secretion (Vitale *et al.* 1995). The increase in cellular Ca^{2+} observed upon CCCP treatment was of such magnitude as to trigger the mechanism responsible for cortical F-actin disassembly. In this regard, it has been suggested that an increase in cytosolic Ca^{2+} might signal the mechanism involved in supplying vesicles to release sites (Von Rüden & Neher, 1993; Kamiya & Zucker, 1994). It can be concluded from the present results that CCCP potentiation of the secretory responses was, as with PMA treatment, the result of translocation of secretory vesicles from the reserve pool to the subplasmalemma area (release-ready vesicle pool) in preparation for exocytosis. As a site of action for these effects, mitochondria were demonstrated to be responsible for the potentiation of the secretory response observed in the presence of CCCP, even when the release of Ca^{2+} from the endoplasmic reticulum was blocked by previous treatment with a cocktail of caffeine, ryanodine and thapsigargin. In addition, exposure of chromaffin cells to a combination of rotenone and oligomycin A, two other mitochondrial inhibitors that do not affect Ca^{2+} mobilization from the endoplasmic reticulum, also caused a potentiation of secretion. The combination of rotenone and oligomycin A not only potentiated the secretory response to high K^+, but also evoked chromaffin cell cortical F-actin disassembly, effects that were also inhibited by chelerythrine (Fig. 8). The inhibition by chelerythrine suggests, again, the involvement of PKC in this process. Two pathways are known to be involved in the control of chromaffin cell cortical F-actin networks during secretion (Trifaró *et al.* 2000). These are the Ca^{2+}–scinderin and the PKC–MARCKS pathway. The Ca^{2+}–scinderin pathway uses Ca^{2+} that enters the cell through voltage-dependent channels during depolarization induced by nicotinic receptor stimulation (Trifaró *et al.* 2000) whereas the PKC pathway seems to be activated during either the release of Ca^{2+} from the endoplasmic reticulum (Zhang *et al.* 1995; Trifaró *et al.* 2000; Rosé *et al.* 2001) or the increase in cell Ca^{2+} observed when mitochondria Ca^{2+} uptake is inhibited, as in this case with CCCP treatment.

In conclusion, our findings suggest that the clearance of cytosolic Ca^{2+} by mitochondria during chromaffin cell stimulation limits the extent of the exocytotic response by preventing excessive stimulation of PKC and enhanced cortical F-actin disassembly. Thus excessive PKC activation would increase the number of secretory vesicles at release sites followed by an increased and perhaps unnecessary secretory response.

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