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RESEARCH ARTICLE

The release of catecholamines to the cytosol and the exocytosis of secretory vesicles triggered by IP_3 in chromaffin cells

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Abstract

The aim of the present study was to investigate the secretory responses elicited by inositol 1,4,5-trisphosphate (IP_3) and their regulation by Ca^{2+} from different sources. Fura-2, carbon fiber amperometry, and plasma membrane capacitance recordings were performed in mouse chromaffin cells to evaluate cytosolic Ca^{2+} changes, catecholamine release, and exocytosis, respectively. Amperometric recordings revealed that IP_3 triggered the continuous release of catecholamines to the cytosol with a plateau shape, either applied independently or in combination with the V-ATPase blocker bafilomycin A1, without exhibiting additive effects, which suggests that V-ATPase blockade might be a potential mechanism of action. The catecholamine release elicited by IP_3 can take place in the absence of cytosolic Ca^{2+}; however, it may be also regulated by it through a bell-shaped mechanism, with the contribution of Ca^{2+} stored in intracellular organelles. Furthermore, plasma membrane capacitance recordings showed that IP_3 could also elicit exocytosis of secretory vesicles with the participation of intracellular organelle Ca^{2+} stores. This exocytosis could be regulated by vesicular or cytosolic Ca^{2+}, as shown in experiments with bafilomycin A1 or the Ca^{2+} chelator BAPTA-AM, respectively, and by kaempferol, an activator of the mitochondrial Ca^{2+} uniporter, suggesting that mitochondria may exert physiologically this Ca^{2+} regulatory mechanism. Therefore, in the IP_3-mediated secretion, Ca^{2+} from different sources control the different steps of catecholamine release from the secretory vesicle to the cytosol and then finally to the extracellular space.

NEW & NOTEWORTHY Inositol 1,4,5-trisphosphate (IP_3) triggers the release of catecholamines from secretory vesicles to the cytosol through a process that may occur in the absence of cytosolic Ca^{2+}, it is biphasically regulated by it and is dependent on Ca^{2+} from intracellular organelles. Additionally, IP_3 triggers the exocytosis of secretory vesicles through a cytosolic and vesicular Ca^{2+} regulatory mechanism that may be physiologically modulated by mitochondria.

amperometry; calcium; capacitance; catecholamine; IP_3

INTRODUCTION

Inositol 1,4,5-trisphosphate (IP_3) is a second messenger that largely contributes to the muscarinic cholinergic responses in the chromaffin cell of the adrenal gland, a neurosecretory cell that mediates the fight and flight mechanisms of stress response. As proved in various cell systems, this molecule activates IP_3 receptors (IP_3Rs) of the endoplasmic reticulum (ER), allowing the release of Ca^{2+} from this organelle through a biphasic Ca^{2+}-dependent process that exhibits a maximum at ~300 nM cytosolic Ca^{2+} (1–3). Whether this Ca^{2+} release shows a correlation with the exocytosis and release of catecholamines is an unresolved question that we investigated in the present study. Here we examined both the amperometric response evoked by IP_3 in permeabilized cells and the exocytosis elicited by muscarinic stimulation in intact cells, as well as the regulation that Ca^{2+} from the cytosol and from IP_3R-expressing intracellular organelles exerts on the secretory process mediated by IP_3. These organelles include the ER and the secretory vesicles; however, it is more plausible that vesicular Ca^{2+} rather than ER IP_3R Ca^{2+} is involved in this process for the following reasons: *1*) vesicular IP_3Rs are placed in the same organelle from which catecholamines are going to be released; *2*) the cortical ER constitutes only 10% of the total ER, and only 10–20% of that cortical ER is colocalized with chromaffin vesicles (4); *3*) in bovine chromaffin cells, the ER contains 15.2%, 19.8%, and

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15.9% of the overall IP_3R_1, IP_3R_2, and IP_3R_3 subtypes, respectively (5), whereas the expression of IP_3R_1, IP_3R_2, and IP_3R_3 subtypes in bovine secretory vesicles is 69.3%, 64.3%, and 58.1%, respectively, of the total amount of each receptor subtype.

Our data show that IP_3 triggers the release of catecholamines from secretory vesicles to the cytosol through a process that does not require cytosolic Ca^{2+} but is biphasically regulated by it with the contribution of Ca^{2+} from IP_3R-expressing organelles, exhibiting a maximum at 300 nM. This is similar to the Ca^{2+} dependence of the IP_3-induced Ca^{2+} release response previously reported in the ER. In addition, IP_3 elicits the exocytosis of secretory vesicles through a mechanism regulated by cytosolic and vesicular Ca^{2+}, which can be modulated physiologically by mitochondria.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (2 mo old) were used for all experiments. Animals were fed with a standard laboratory diet, with water and food provided ad libitum.

Cell Culture of Mouse Chromaffin Cells

Chromaffin cells of the adrenal glands were isolated and maintained in culture with the method previously performed in our laboratory (6). Briefly, adrenal glands removed from the animal euthanized by cervical dislocation were incubated for 25 min in a solution with papain (25 IU/mL) (Merck) at 37°C. After this time, they were washed with Locke's solution (in mM: 154 NaCl, 5.6 KCl, 3.6 $NaHCO_3$, 5 HEPES, 5.6 glucose at pH 7.2), and then with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Then mechanical digestion was performed by passing the tissue through a micropipette tip until a homogeneous suspension was obtained. Finally, cells were plated on glass coverslips previously treated with poly-L-lysine (0.1 mg/mL). They were kept in an incubator with 95% air and 5% CO_2.

Amperometry

Carbon fiber electrodes were prepared by cannulating a 10-μm-diameter carbon fiber in polyethylene tubing (diameter: outer, 1 mm; inner, 0.5 mm). The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp headstage and backfilled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The carbon fiber electrode was gently resting on one side of the cell under study. Amperometric currents were recorded with an EPC-10 amplifier and PULSE software running on a PC. The sampling rate was 14.5 kHz. The sensitivity of the electrodes was routinely monitored before and after experiments with 50 μM of adrenaline as standard solution. Only fibers that rendered 200–300 pA of current increment with a pulse of 50 μM adrenaline were used for the experiments. The tip of the fiber was recut at the start of each experiment and calibrated again.

Cells were perfused with a solution containing (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. Then, a solution of the following composition was perfused (in mM): 139 K-glutamate, 0.2 EGTA, 20 PIPES, 2 ATP, and 3 $MgCl_2$ (*solution A*). In the experiments that required permeabilization of the cells, a 10-s pulse of digitonin at 20 μM dissolved in *solution A* was applied. Different Ca^{2+} concentrations were added to *solution A* to reach the final free Ca^{2+} concentrations (from 100 nM to 10 μM) to be further tested. The Ca^{2+}-EGTA calculator v1.2 using constants from NIST database no. 46 v8 was used to calculate the amount of Ca^{2+} required to obtain the value of the free Ca^{2+} concentration.

In the control experiments (see Fig. 3), the different Ca^{2+} concentration solutions were perfused from the start of the experiment, 20 s before each IP_3 pulse, until the next Ca^{2+} concentration was applied. Instead, in the Ca^{2+}-depleted experiments, the perfusion continued only until 5 s after each IP_3 pulse. During the entire course of these latter experiments, a solution containing 0.2 mM EGTA and 1 μM thapsigargin was perfused between pulses (see Fig. 4). The schemes of both protocols are shown in Fig. 3*B* and Fig. 4*B*, respectively.

Electrophysiological Recordings of "Patch Clamp"

The perforated patch configuration of the patch-clamp technique was used. The holding potential (V_h) was −80 mV. To perform plasma membrane capacitance (C_m) recordings after muscarinic stimulation, the extracellular solution had the following composition (in mM): 2 $CaCl_2$, 145 NaCl, 5.5 KCl, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4 (NaOH). The composition of the intracellular solution was (in mM): 145 K-glutamate, 8 NaCl, 1 $MgCl_2$, and 10 HEPES, pH 7.2. Amphotericin B at 0.5 mg/mL was included in this solution, obtained from a stock of 50 mg/mL prepared every day in dimethyl sulfoxide and protected from light. The pipettes were immersed in amphotericin-free solution for several seconds and filled in the back with intracellular solution. The perfusion system for the application of drugs consisted of a homemade system that exchanged the solutions very rapidly (7). This system was positioned ∼50 μm away from the cell. Repetitive pulses of acetyl-β-methylcholine chloride (methacholine) were applied every 5 min until stable responses were obtained, and in that moment the different compounds to be tested were perfused during 90 s before the next pulse of methacholine and then during the following pulses of methacholine until a stable and maximum effect was achieved (Fig. 5 and Fig. 6).

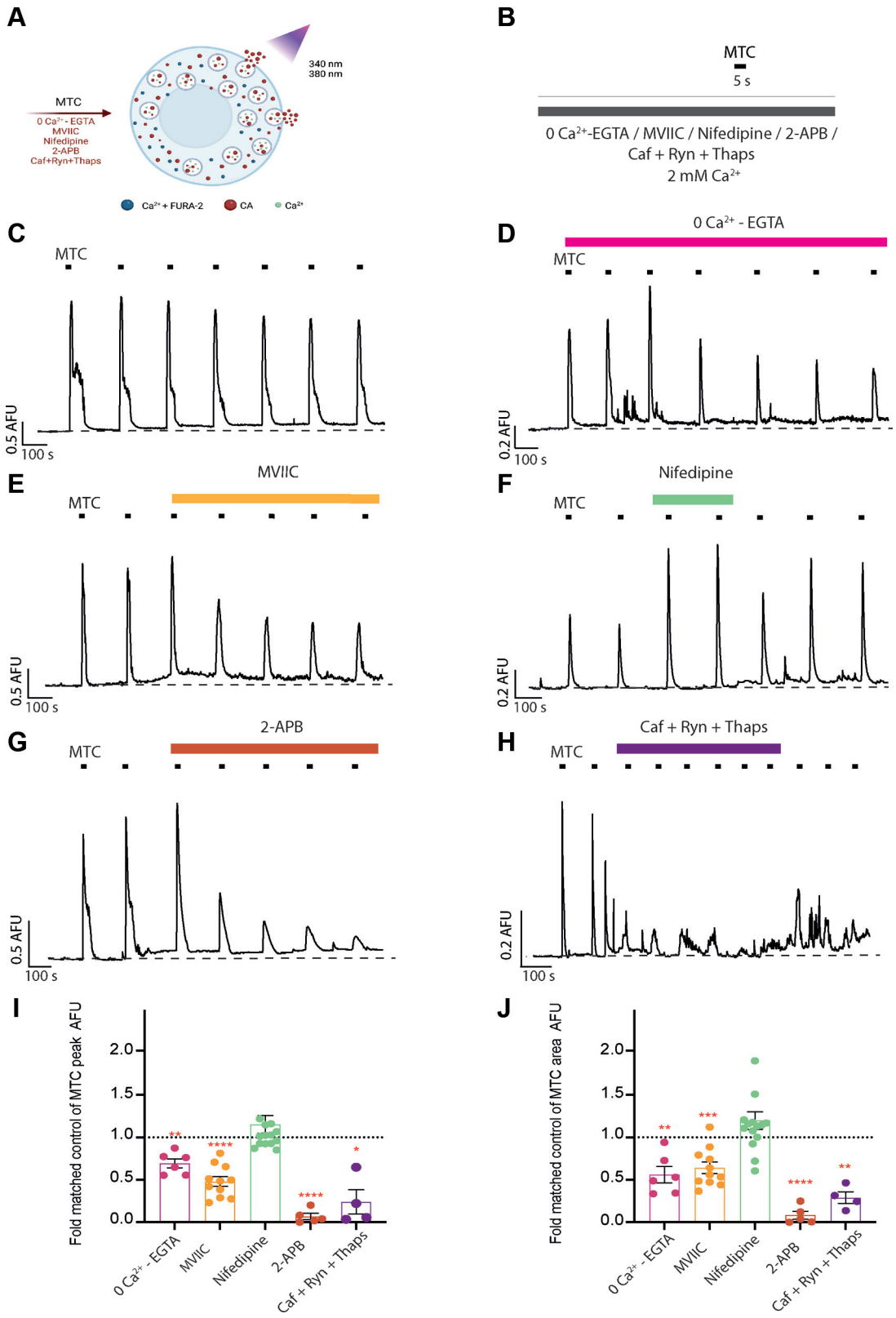
The electrophysiological measurements were carried out with an EPC-10 amplifier and the PULSE program (HEKA Elektronik, Germany). Borosilicate pipettes of 2–3 MΩ, partially covered with wax and polished, were used. After the formation of the seal and the perforation, only those recordings in which the access resistance of the pipette and the leakage current were less than 20 MΩ and 20 pA, respectively, were accepted. Changes in the C_m as an index of exocytosis were recorded. These changes were estimated by the Lindau–Neher technique (8). A sinusoidal wave of 1 kHz and 70 mV of peak-to-peak amplitude over a fixation potential of −80 mV was applied.

Fluorometric Recordings of Fura-2

Cells were incubated in extracellular medium with Fura-2 AM (1 μM) in DMEM without serum, in the dark and during 10 min at 37°C in a culture chamber. After that time, a

washout in extracellular medium without the Ca^{2+} probe was performed during another 10 min at room temperature.

An epifluorescence microscope (Nikon Eclipse TE2000) with a monochromator (Cairn Research, United Kingdom), coupled to a xenon arc lamp, was used. The monochromator was controlled by Metafluor software (Molecular Devices, United States). An oil immersion objective Nikon S Fluor 40X [numerical aperture (NA) 1.3] was employed. Alternative



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excitation wavelengths of 340 and 380 nm at a frequency of 2 Hz were used. The emission filter was a 510/84 (Semrock, United States).

Experimental Design and Statistical Analysis

The number of data n refers to the number of cells. Data are given as means ± SE. A Kolmogorov–Smirnov normality test was first performed. Then, Student t, Mann–Whitney U, Wilcoxon, or ANOVA signed-rank tests were used as indicated in each experiment. The parametric Student t test is reported as t value, degrees of freedom, and exact P value. In the case of nonparametric tests, Wilcoxon matched-pairs signed-rank test is shown as the sum of single ranks (W), number of pairs, and exact P value. Finally, the ordinary one-way ANOVA is reported as F values and exact P value. Statistical analysis was performed by GraphPad Prism. Differences were accepted as significantly different when the P value was <0.05, 0.01, 0.001, and 0.0001. All data values are shown in bar charts with overplotted individual values.

Chemical Compounds

Bafilomycin A1 (PubChem CID: 72311), thapsigargin (PubChem CID: 446378) and ryanodine (PubChem CID: 11317883) were purchased from Tocris; IP_3 (PubChem CID: 329815468), BAPTA-AM (PubChem CID: 24890502), acetyl-β-methylcholine chloride (PubChem CID: 24277821), kaempferol (PubChem CID: 57651711), 2-aminoethyl diphenylborinate (2-APB) (PubChem CID: 57654145) and caffeine (PubChem CID: 24277682) were purchased from Merck.

RESULTS

The Ca^{2+} Signal and Exocytosis Evoked by Muscarinic Stimulation Are Mediated by Extracellular Ca^{2+} Together with Ca^{2+} from Intracellular Organelles through an IP_3-Mediated Process

The aim of the present study was to investigate the secretory response triggered by IP_3 and its regulation by Ca^{2+} from different sources. Because of the lack of permeability of IP_3 through the plasma membrane, we activated the muscarinic receptor to trigger the formation of IP_3 in intact cells, using methacholine (100 μM). We characterized the Ca^{2+} signal and exocytosis elicited by this agonist in mouse chromaffin cells incubated with the Ca^{2+} probe Fura-2 AM. The scheme and protocol of the experiment are shown in Fig. 1, *A* and *B*. Pulses of methacholine of 5-s duration were applied every 5 min. Control responses were first obtained, which exhibited a decay of the area of 17.5 ± 9.5% (n = 9) (Fig. 1*C*). Removal of Ca^{2+} from the extracellular solution caused a peak and area Ca^{2+} signal decrease, given responses that represented 0.7 ± 0.05-fold and 0.6 ± 0.09-fold with respect to their control [$t(5)$ = 5.78, P = 0.0022 and $t(5)$ = 4.6, P = 0.006], respectively (n = 6) (Fig. 1, *D*, *I*, and *J*). The contribution of voltage-dependent Ca^{2+} channels (VDCCs) to the muscarinic evoked response was also investigated by perfusing the cells with ω-conotoxin MVIIC (MVIIC), a blocker of N- and P/Q-type Ca^{2+} channels (Fig. 1*E*), and nifedipine (Fig. 1*F*), a blocker of L-type Ca^{2+} channels (both at 3 μM). The methacholine-evoked peak and area Ca^{2+} signals were diminished by MVIIC, giving signals that corresponded to 0.48 ± 0.06 [$t(10)$ = 9.3, P < 0.0001]- and 0.64 ± 0.07 [$t(10)$ = 5.3, P = 0.0004]-fold with respect to the matched control, respectively, reflecting the participation of N- and P/Q-type channels in the response (n = 11). Nifedipine increased or decreased the Ca^{2+} signal in the different cells, although statistical differences were not significant, given on average values of the peak and area Ca^{2+} signals, which corresponded, respectively, to 1.1 ± 0.1 [$t(14)$ = 1.5, P = 0.15]- and 1.2 ± 0.1 [$t(14)$ = 2, P = 0.073]-fold with respect to the matched control (n = 15) (Fig. 1, *I* and *J*). The increased response in some cells might be due to the coupling of L-type Ca^{2+} channels with Ca^{2+}-dependent K^+ channels, resulting in a net depolarization of the membrane with the subsequent increase of the Ca^{2+} signal, as previously shown in these cells (9, 10). In summary, these data indicate that N-, P/Q-, and L-type Ca^{2+} channels contribute to the extracellular Ca^{2+} signal triggered by muscarinic stimulation.

The contribution of IP_3 to that response was investigated by perfusing an IP_3R antagonist, the compound 2-APB (100 μM), after the methacholine control pulses and then during the application of successive methacholine pulses (Fig. 1*G*). The blockade of the response elicited by 2-APB left signals of 0.07 ± 0.04 [$t(4)$ = 25.83, P < 0.0001]- and 0.14 ± 0.05 [$t(4)$ = 19.6, P < 0.0001]-fold with respect to the control of the peak and area responses (n = 5), respectively.

The ER as well as secretory vesicles (11) have been shown to express IP_3Rs, ryanodine receptors, and the SERCA pump to control Ca^{2+} dynamics. Therefore, the contribution of Ca^{2+} from intracellular stores was evaluated by perfusing cells with a cocktail of 10 mM caffeine, an agonist of the ryanodine receptor that allows the extrusion of Ca^{2+} from the organelle, 10 μM ryanodine, which maintains the ryanodine receptor in a semiconductance state after receptor activation by caffeine, also promoting the depletion of Ca^{2+} from the organelle, and 1 μM thapsigargin, an inhibitor of ER and secretory vesicle ATPases that avoids Ca^{2+} replenishment in the organelle (12)

Figure 1. The Ca^{2+} signal evoked by muscarinic stimulation is mediated by extracellular Ca^{2+} together with mobilization of Ca^{2+} from intracellular organelles through an inositol 1,4,5-trisphosphate (IP_3)-mediated process. Cells were incubated with Fura-2 AM, and the cytosolic Ca^{2+} signal was recorded. Pulses of 5-s duration of 100 μM methacholine (MTC) were applied every 5 min. *A* and *B*: scheme and protocol of the experiment, respectively. 2-APB, 2-aminoethyl diphenylborinate; 0Ca-EGTA, 5 mM EGTA; CA, catecholamine; Caf, caffeine; MTC, methacholine; MVIIC, ω-conotoxin MVIIC; Ryn, ryanodine; Thaps, thapsigargin. *C*–*H*: representative cytosolic Ca^{2+} recordings of the MTC-mediated Ca^{2+} signal under control conditions (*C*), in the absence of extracellular Ca^{2+} (*D*; n = 6), and after perfusion with 3 μM MVIIC (*E*; n = 11), 3 μM nifedipine (*F*; n = 15), 100 μM 2-APB (*G*; n = 5), or a cocktail of 10 mM Caf, 1 μM Ryn, and 1 μM Thaps (*H*; n = 4). Once MTC control responses were stable, the different compounds were applied 90 s before the next MTC pulse and then during the rest of the experiment, until maximum and stable effect was achieved. AFU, Arbitrary Fluorescence Unit. *I* and *J*: dot plots of the fold times of the peak (*I*) or the area (*J*) of the cytosolic Ca^{2+} transients under each condition with respect to their own control. Statistical test: one-sample t test (*P < 0.05; **P < 0.01, ***P < 0.001, ****P < 0.0001). Figure created with a licensed version of BioRender.com.

(Fig. 1*H*). This cocktail blocked the peak and area, remaining signals corresponded to 0.05 ± 0.01 [$t(3) = 5.35$, $P = 0.0128$]- and 0.23 ± 0.09 [$t(3) = 10.4$, $P = 0.0019$]-fold with respect to the matched control, respectively ($n = 4$). In summary, extracellular Ca^{2+} together with Ca^{2+} released from intracellular organelles contribute to build up the muscarinic-elicited Ca^{2+} signal in mouse chromaffin cells.

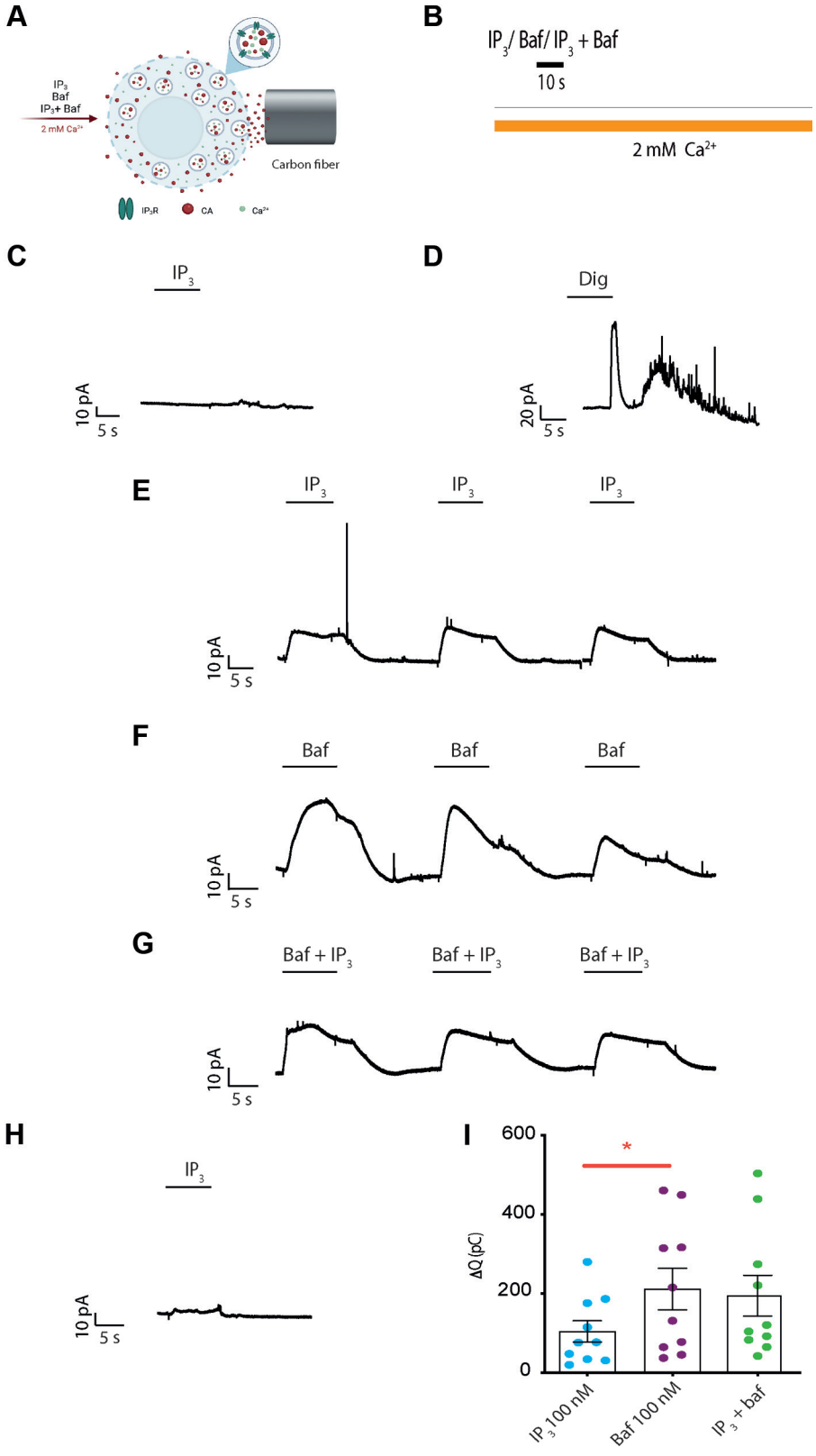

Figure 2. Inositol 1,4,5-trisphosphate (IP_3) triggers the release of catecholamines from secretory vesicles to the cytosol: the V-ATPase blockade as a potential mechanism. Catecholamine release was recorded with a 10-μm carbon fiber electrode gently touching the cell membrane. *A* and *B*: scheme and protocol of the experiment, respectively. Baf, bafilomycin A1; CA, catecholamine; IP_3R, IP_3 receptor. *C* and *H*: amperometric recordings of the carbon fiber electrode obtained in the absence of cell after the application of 10-s pulses of 2 μM IP_3 at the beginning (*C*) and at the end (*H*) of the experiment. *D*: amperometric recordings obtained from a cell permeabilized with a 10-s pulse of 20 μM digitonin (Dig). *E*–*G*: amperometric recordings obtained from the same cell stimulated with 10-s pulses of 2 μM IP_3 (*E*), 100 nM Baf (*F*), and 2 μM IP_3 + 100 nM Baf (*G*). *I*: dot plots of the charge of the amperometric recordings (Δ*Q*) obtained under each condition ($n = 10$). *$P < 0.05$. *A* created with a licensed version of BioRender.com.

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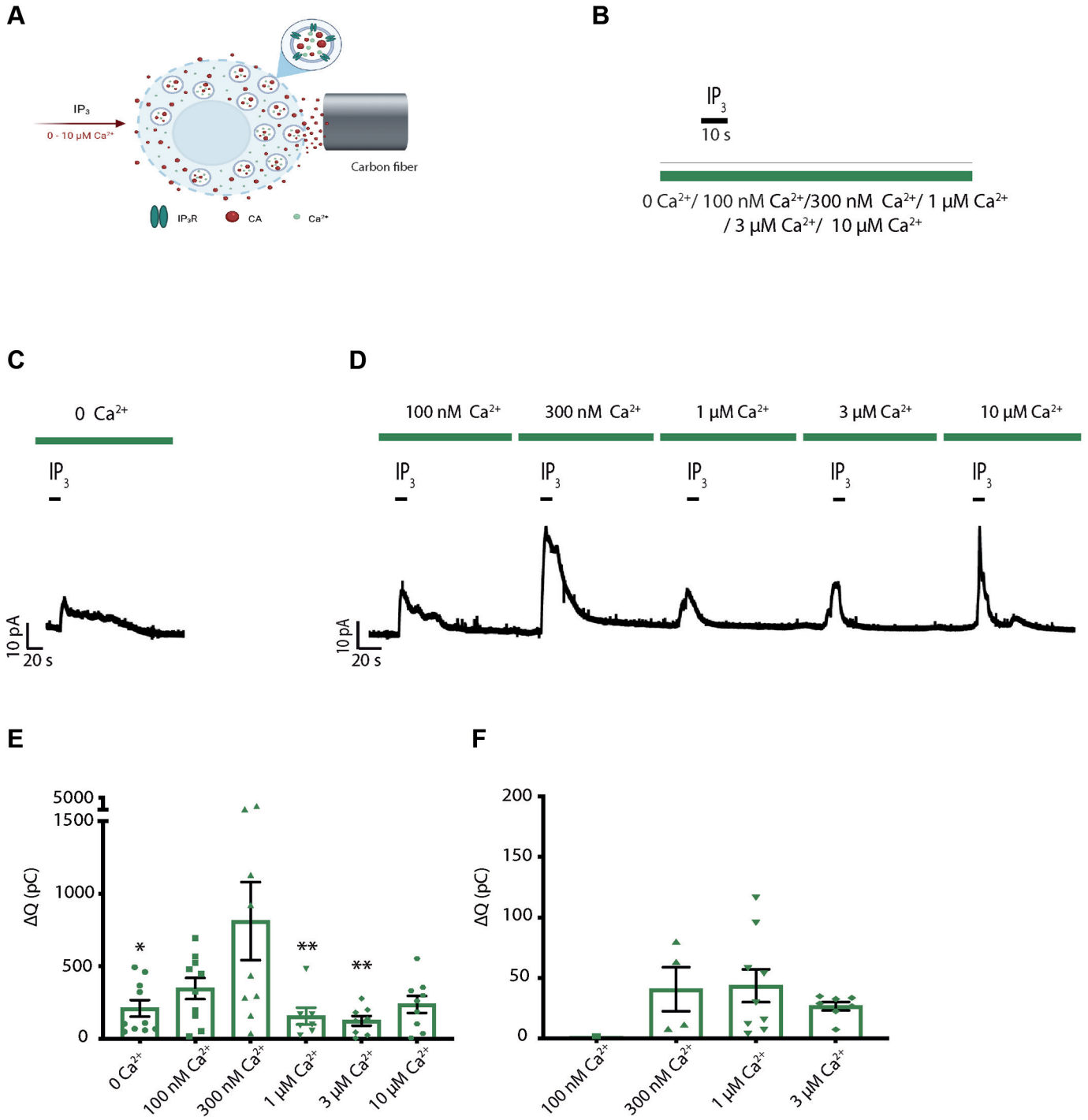


Figure 3. Inositol 1,4,5-trisphosphate (IP_3) triggers the release of catecholamines from secretory vesicles to the cytosol through a process that may occur in the absence of cytosolic Ca^{2+} and is biphasically regulated by it. Catecholamine release was recorded with a 10-µm carbon fiber electrode gently touching the cell membrane. Cells were permeabilized with a 10-s pulse of 20 µM digitonin and stimulated with 10-s pulses of 2 µM IP_3 every 5 min. *A* and *B*: scheme and protocol of the experiment. CA, catecholamine; IP_3R, IP_3 receptor. *C*: representative recording of the amperometric catecholamine release obtained after a pulse of IP_3 was applied in the absence of Ca^{2+} ($n = 10$). *D*: pulses of IP_3 were applied together with different free Ca^{2+} concentrations, ranging from 100 nM to 10 µM ($n = 10$). *E*: dot plots of the charge of the catecholamines released by IP_3 (ΔQ) at the different Ca^{2+} concentrations ($n = 10$). Values achieved at 0, 1 µM and 3 µM Ca^{2+} concentration compared with 300 nM exhibited significant statistical differences. Statistical test: ordinary one-way ANOVA, Tukey's multiple comparisons test (*$P < 0.05$, **$P < 0.01$). *F*: dot plots of the charge of the catecholamines released by the different Ca^{2+} concentrations tested in response to Ca^{2+} (100 nM, $n = 1$; 300 nM, $n = 4$; 1 µM, $n = 9$; 3 µM, $n = 7$). No significant statistical differences among Ca^{2+} concentrations were achieved. *A* created with a licensed version of BioRender.com.

IP_3 Triggers the Release of Catecholamines from Secretory Vesicles to the Cytosol: V-ATPase Blockade as a Potential Mechanism

We investigated the IP_3-mediated secretory response by recording the catecholamine release in permeabilized mouse chromaffin cells with the high-resolution carbon fiber amperometric technique. A scheme of the experimental configuration and the protocol of the experiment are shown in Fig. 2*A* and *B*, respectively. First, the signal of the electrode (in the absence of cell) was recorded after perfusion of a 10 s pulse of 2 μM IP_3, which did not exhibit any response (Fig. 2*C*). Then cells were permeabilized with 10 s of *solution A* containing 20 μM digitonin to allow the entry of IP_3 to the cytosol. A burst of amperometric spikes could be recorded (Fig. 2*D*). Later on, cells were stimulated three times with 10-s pulses of IP_3 giving responses that were similar in amplitude and shape, which consisted in a continuous line with a plateau (Fig. 2*E*). This experiment suggests that IP_3 is releasing catecholamines from the secretory vesicles to the cytosol, and these free catecholamines are afterwards exiting the cell through the pores created by digitonin in the plasma membrane.

To investigate the mechanism of catecholamine release from the secretory vesicles by IP_3, an inhibitor of V-ATPase, bafilomycin A1, was also used since it was previously shown that alkalinization displaces catecholamines from the vesicular compartment into the cytosol in bovine chromaffin cells (Ref. 13, Fig. 6b). Therefore, three pulses of bafilomycin A1 (100 nM) were then applied (Fig. 2*F*), also eliciting the release of plateau-shaped catecholamines, and finally three pulses of IP_3 in the presence of bafilomycin A1 (10-s duration) evoked a similar response (Fig. 2*G*). The effect of both compounds applied together did not give additive results (Fig. 2*I*), indicating that a possible mechanism of the IP_3-elicited release of catecholamines might be the inhibition of V-ATPase (13). The signal of the electrode in the presence of IP_3 was also recorded at the end of the experiment, showing the absence of effect (Fig. 2*H*). Dot plots of the values achieved under the different conditions are shown in Fig. 2*I*.

IP_3 Triggers the Release of Catecholamines from Secretory Vesicles to the Cytosol through a Process That May Occur in the Absence of Cytosolic Ca^{2+} and Is Biphasically Regulated by It

The regulation by Ca^{2+} of the observed release of catecholamines triggered by IP_3 was then investigated. As in Fig. 2, experiments were performed by recording the transient current of oxidation of the catecholamines released with a carbon fiber electrode. The scheme and protocol of the experiment are shown in Fig. 3, *A* and *B*, respectively. First, a pulse of IP_3 was applied in the absence of free Ca^{2+}, showing a plateau-shaped amperometric signal without spikes (Fig. 3*C*). Then the possibility that cytosolic Ca^{2+} might be contributing to regulate that process biphasically, in a similar manner as it does by acting on IP_3Rs in the ER, was investigated by challenging the cell with free Ca^{2+} concentrations ranging from 100 nM to 10 μM. Representative recordings of the amperometric transients elicited are shown in Fig. 3*D*. A bell-shaped Ca^{2+}-dependent response exhibiting a peak at 300 nM cytosolic Ca^{2+} was achieved [$F(5.53) = 4.183$, $P = 0.0028$] (Fig. 3*E*). It is interesting to note that the Ca^{2+} itself provided for 20 s before the IP_3 pulse triggered a small signal previously to the main response. The charge of the catecholamines released at the different Ca^{2+} concentrations during these signals did not exhibit significant statistical differences and occurred mainly between 300 nM and 3 μM Ca^{2+} (Fig. 3*F*), indicating that a calcium-induced calcium release (CICR) mechanism might be involved in this process as confirmed in the experiments of Fig. 4, in which cells were depleted of free Ca^{2+} from the intracellular organelles, and those signals under this condition disappeared.

In summary, the amperometric data here obtained in permeabilized cells have allowed us to prove that IP_3 triggers catecholamine release from secretory vesicles to the cytosol through a pathway that does not require cytosolic Ca^{2+} but it is biphasically regulated by it.

IP_3 Triggers the Release of Catecholamines from Secretory Vesicles to the Cytosol through a Process That Is Dependent on Ca^{2+} from Intracellular Organelles

To check the possibility that Ca^{2+} from intracellular organelles expressing IP_3Rs might be contributing to the bell-shaped Ca^{2+} regulation of the IP_3-mediated catecholamine release response, cells were treated with 1 μM thapsigargin in the absence of Ca^{2+} during 15 min before the beginning and then during the whole experiment, to deplete those organelles of free Ca^{2+} (ER and secretory vesicles). In addition, this would also help to determine the presence of a CICR mechanism mediating the release of catecholamines by Ca^{2+} itself shown in Fig. 3*F*.

The scheme and protocol of the experiment are shown in Fig. 4, *A* and *B*, respectively. Representative amperometric recordings of the IP_3 stimulation in the absence of Ca^{2+} and then with increasing Ca^{2+} concentrations in cells treated with EGTA and thapsigargin are displayed in Fig. 4, *C* and *D*, respectively. The charges of the catecholamines released at the various Ca^{2+} concentrations perfused are shown in the dot plots of Fig. 4*E* (pink bars), not showing significant statistical differences. These results show that cytosolic Ca^{2+} is not regulating the IP_3-mediated response when the intracellular organelles are depleted of free Ca^{2+} [$F(5.53) = 1.925$, $P = 0.1056$], reflecting that the IP_3-elicited catecholamine release process occurs through a CICR pathway. In addition, a significant reduction of catecholamine secretion was only obtained in the absence of Ca^{2+} and at basal Ca^{2+} concentrations (100 nM) with respect to their control situation (green bars, data from Fig. 3*E*), showing that Ca^{2+} from intracellular organelles contributes to the total amount of catecholamine release under these conditions [$W(10) = -47$, $P = 0.014$ and $t(9) = 3.114$, $P = 0.012$, respectively].

IP_3 Triggers the Fusion of Secretory Vesicles with the Plasma Membrane through a Process That Is Dependent on Ca^{2+} from Intracellular Organelles, Is Biphasically Regulated by Cytosolic Ca^{2+}, and Is Inhibited by Vesicular Ca^{2+}

We next investigated the exocytotic response elicited by IP_3 by recording C_m in voltage-clamped cells. In this way, the contribution of VDCC to the response was avoided. The

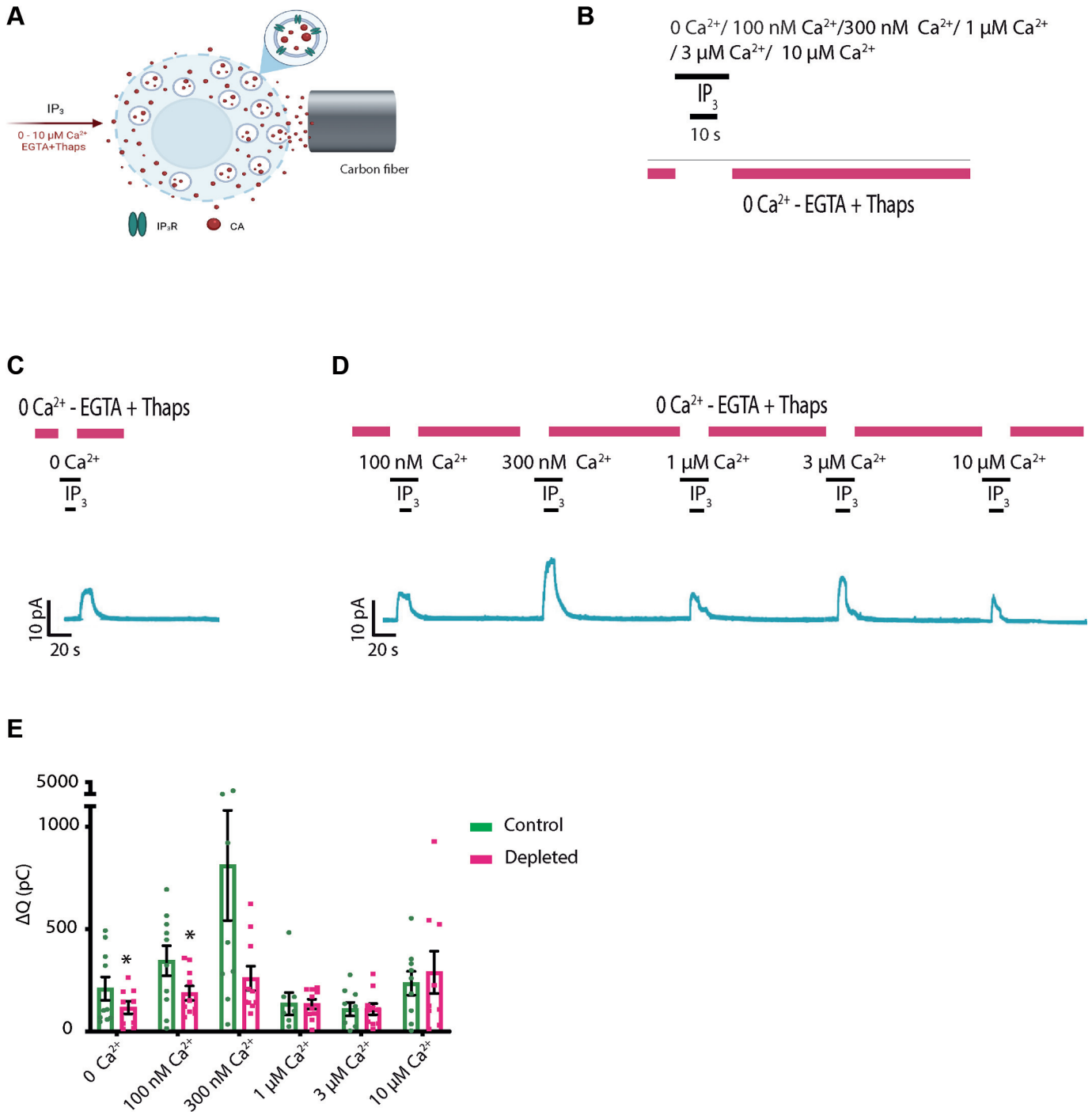
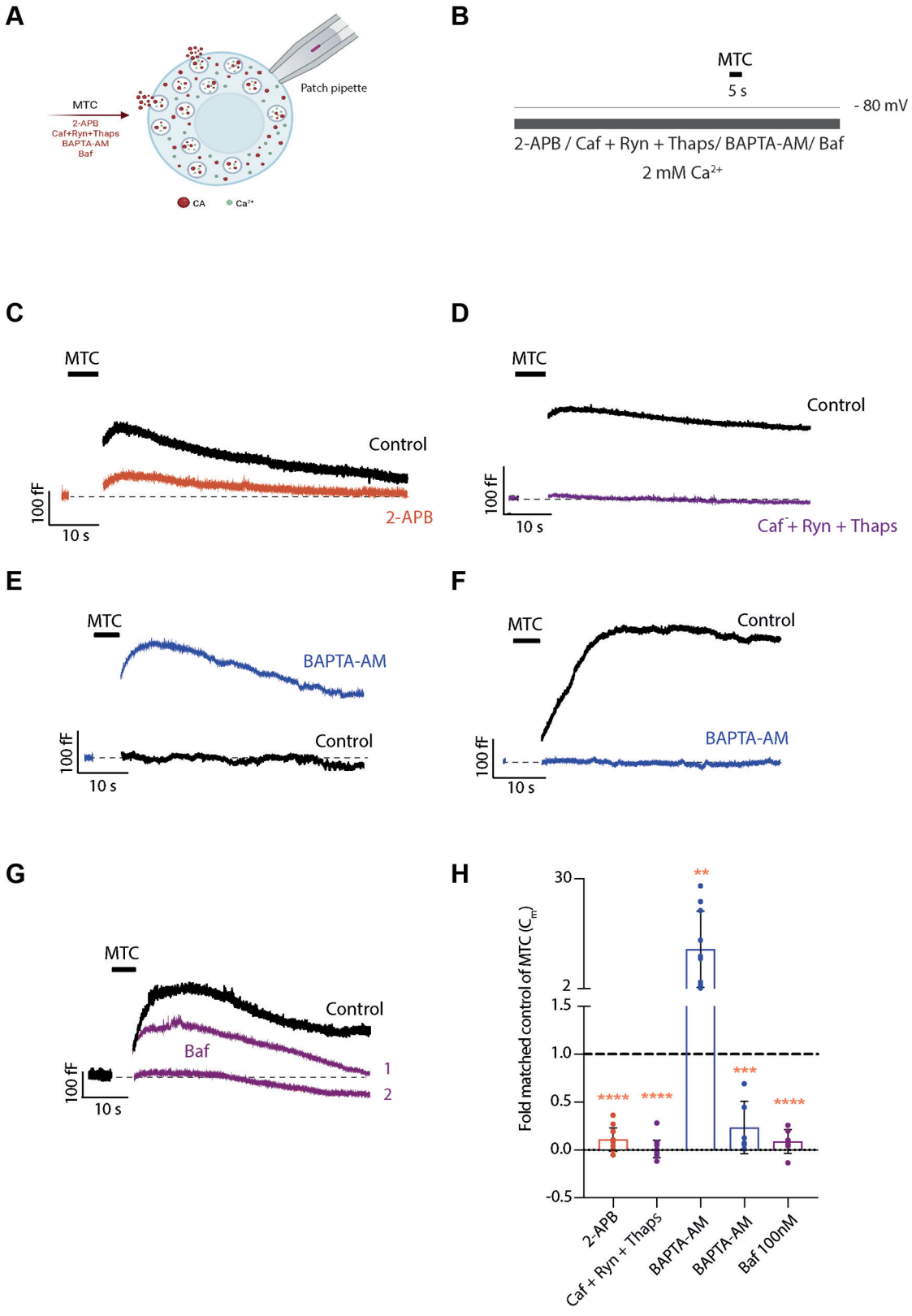


Figure 4. Inositol 1,4,5-trisphosphate (IP_3) triggers the release of catecholamines from secretory vesicles to the cytosol through a process that is dependent on Ca^{2+} from intracellular organelles. Catecholamine release was recorded with a 10-µm carbon fiber electrode gently touching the cell membrane. Cells were perfused with the Ca^{2+} depletion solution, which contains 5 mM EGTA and 1 µM thapsigargin (Thaps). *A* and *B*: scheme and protocol of the experiment. CA, catecholamine; IP_3R, IP_3 receptor. *C* and *D*: representative recordings of the amperometric catecholamine release obtained after a 10-s pulse of IP_3 was applied in the absence of Ca^{2+} (*C*) and in the presence of 100 nM to 10 µM of Ca^{2+} for 5 s before, during, and 5 s after the IP_3 pulse (*D*) ($n = 10$). *E*: dot plots of the charge of the catecholamines released by IP_3 (ΔQ) in depleted cells at the different Ca^{2+} concentrations perfused (pink bars). Statistical test: ordinary 1-way ANOVA, Tukey's multiple comparisons test, not significant (ns). In addition, the dot plots of the charge of the catecholamines released at the different Ca^{2+} concentrations in control cells (green bars, results from Fig. 3*E*) are displayed for comparison. Statistical test: Wilcoxon matched-pairs signed-rank test and paired *t* test (*$P < 0.05$). *A* created with a licensed version of BioRender.com.

muscarinic exocytotic response in mouse chromaffin cells is mediated by a muscarinic receptor of the M1 type (14). The scheme and protocol of the experiment are shown in Fig. 5, *A* and *B*, respectively. Exocytosis was triggered by methacholine in 120 out of 138 cells tested, ranging from 13 to 1,758 fF. The contribution of the IP_3R-expressing intracellular organelle Ca^{2+} sources to the exocytosis was investigated by perfusing 2-APB (100 µM), which decreased C_m

from 168.9 ± 26.7 fF to 17.4 ± 7 fF, leaving responses that represented 0.11 ± 0.03-fold of their control [$t(11) = 24.9$, $P < 0.0001$] (Fig. 5, *C* and *H*). Exocytosis was abolished by a cocktail of caffeine (10 mM), ryanodine (1 μM), and thapsigargin (1 μM), which reduced C_m from 208 ± 53.8 fF to −1.2 ± 3.4 fF, being 0.01 ± 0.04-fold of their matched control in the 17 cells tested (Fig. 5, *D* and *H*). This reflects that exocytosis evoked by IP_3 is dependent on intracellular Ca^{2+} stores [$t(16) =$



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23.76, $P < 0.0001$]. Therefore, intracellular organelles contribute to the trigger of the muscarinic-mediated exocytotic response.

Additional experiments were also performed to prove a cytosolic Ca^{2+} regulatory pathway controlling the IP_3-mediated exocytosis in intact cells. Here we posed the hypothesis that the reduced exocytosis initially achieved in some cells (~20 pF) could be due to tonic inhibition by Ca^{2+} of IP_3Rs, similar to the Ca^{2+} dependence previously reported for IP_3Rs. To evaluate that possibility, the fast Ca^{2+} chelator BAPTA-AM (50 μM) was tested on the exocytosis elicited by methacholine, recorded as C_m increments. The exocytotic response increased in 11 cells, from 12.7 ± 6.8 fF to 78.6 ± 32.5 fF, these signals being 11.7 ± 3.12-fold with respect to the control exocytosis [$t(9) = 3.4$, $P = 0.0074$] (Fig. 5, *E* and *H*). This astonishing finding, the facilitation of exocytosis by a Ca^{2+} chelator, implies that Ca^{2+} was inhibiting the IP_3-mediated response, probably the IP_3Rs, preventing the release of Ca^{2+} that mediated the muscarinic exocytotic response. However, in the rest of the cells with larger exocytotic initial values, BAPTA-AM decreased the response. On average, exocytosis elicited by methacholine was diminished by the chelator from 212.2 ± 47.5 fF to 35.7 ± 11.4 fF ($n = 6$), leaving a 0.23 ± 0.1-fold response with respect to the initial amount of exocytosis [$t(5) = 6.9$, $P = 0.00$] (Fig. 5, *F* and *H*). These data offer evidence of the cytosolic Ca^{2+} regulation of the IP_3-mediated exocytotic response after muscarinic stimulation in intact cells.

We performed additional experiments to investigate whether vesicular Ca^{2+} might contribute to the regulation of exocytosis elicited by IP_3R stimulation. Bafilomycin A1 was used to evaluate this issue (15). Here we show that exocytosis was abolished after bafilomycin A1 was applied for 2 s before the methacholine pulse. Control exocytosis of 254.9 ± 70 fF and 158 ± 44.2 fF was fully blocked, respectively, by two or three pulses of 100 nM ($n = 7$) [$t(8) = 19.4$, $P < 0.0001$] (Fig. 5, *G* and *H*) or one or two pulses of 300 nM bafilomycin A1 [$t(4) = 18.3$, $P < 0.0001$] (data not shown), respectively ($n = 5$). This might be due to a Ca^{2+} concentration increase around IP_3Rs elicited by bafilomycin A1 to values that inhibit those receptors. Thus, Ca^{2+} released from secretory vesicles is able to inhibit the IP_3-elicited exocytotic response.

Mitochondria Contribute Physiologically to Regulation by Cytosolic Ca^{2+} of the IP_3-Triggered Exocytotic Response

Physiologically, the IP_3-mediated exocytosis response could be controlled by mitochondria. Thus, we also evaluated the role that mitochondria might have in regulating this process triggered by muscarinic receptor stimulation. In chromaffin cells, mitochondria can take up Ca^{2+} released from the ER either when ryanodine receptors are activated with caffeine (16) or through IP_3R stimulation with histamine (17). Indeed, one-third of the cortical mitochondria colocalize with exocytotic sites in chromaffin cells (4). Thus, as removal of Ca^{2+} with BAPTA-AM increased the exocytosis elicited by IP_3R stimulation, we wanted to test whether favoring the Ca^{2+} uptake by mitochondria might also remove the inhibition by Ca^{2+} of IP_3Rs.

To achieve this goal, cells were perfused with the mitochondria Ca^{2+} uniporter activator (MCU) kaempferol (KPF) (10 μM) before and during the methacholine pulse (18, 19). The scheme and protocol of the experiment are shown in Fig. 6, *A* and *B*, respectively. As happened with BAPTA-AM, two types of methacholine-elicited exocytotic responses were obtained with KPF. The response could be enhanced in some cells, from 40.4 ± 8 fF to 244 ± 55 fF ($n = 11$), which represented 4.6 ± 0.65-fold with respect to the initial exocytosis [$t(10) = 5.5$, $P = 0.0002$] (Fig. 6, *C* and *G*). In other cells with larger initial exocytosis, KPF decreased the response from 315 ± 45 fF to 123 ± 28 fF ($n = 13$), which represented a response of 0.36 ± 0.03-fold with respect to their matched control [$t(12) = 18.8$, $P < 0.0001$] (Fig. 6, *D* and *G*). Also, both types of responses could be investigated in the same cell, by varying the length of the stimulus. In this way, KPF could increase the response elicited by 50-ms pulses of methacholine and decrease it when 5-s pulses of this agonist were applied ($n = 3$) (Fig. 6, *E* and *F*, respectively), showing that mitochondria contribute to the regulation by Ca^{2+} of the IP_3-elicited response.

DISCUSSION

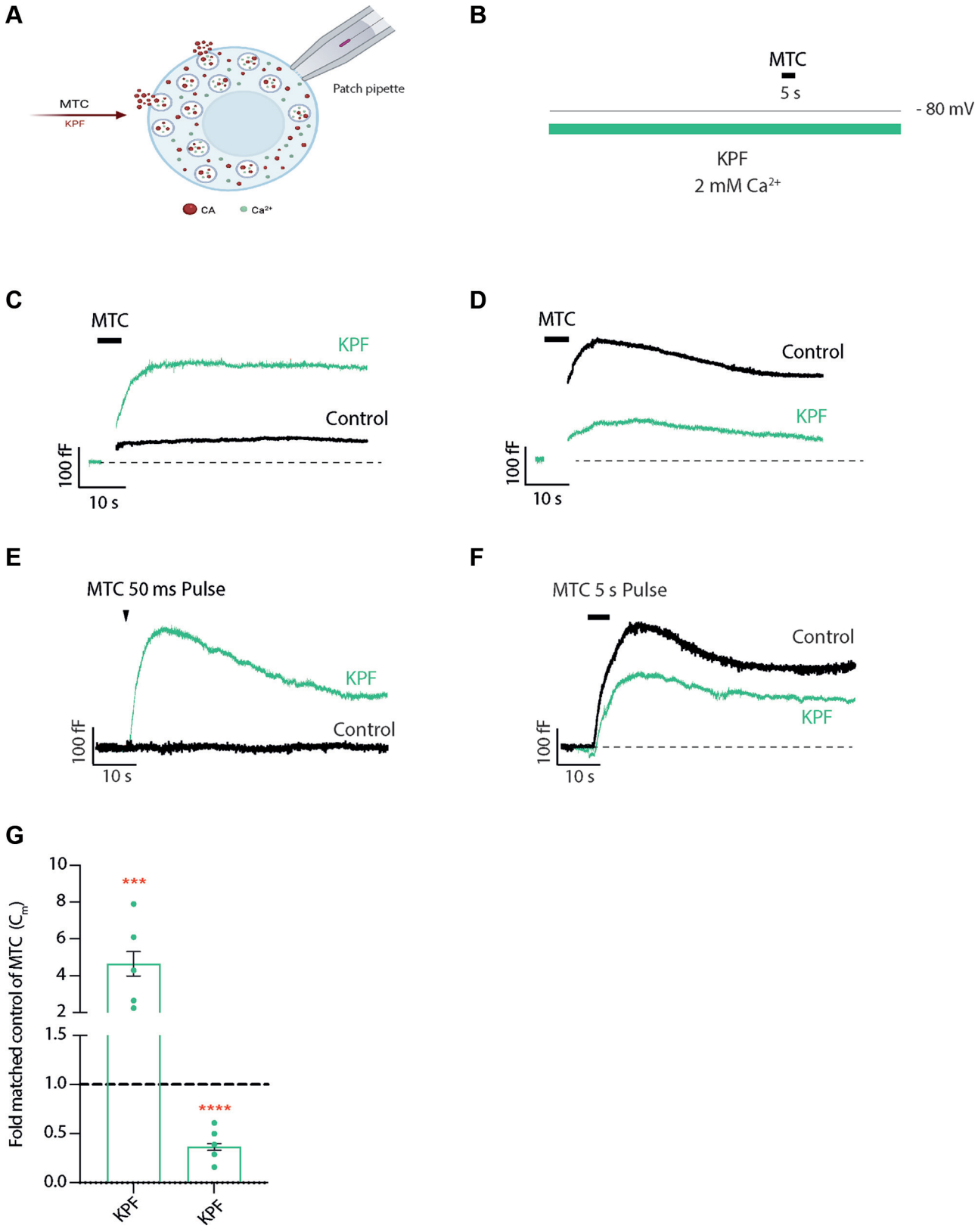
We have here investigated the regulation by Ca^{2+} from different sources (cytosol and intracellular organelles) of the IP_3-evoked secretory process. A remarkable finding of the present study is that IP_3 triggers the release of catecholamines from secretory vesicles. It is interesting to note that cytosolic catecholamines are strictly maintained at a defined level in bovine chromaffin cells (20), being 10% of the total cytosolic catechol concentration, which is 50–500 μM. However, the physiological stimuli that trigger the release of catecholamines to the cytosol remained unknown.

The amperometric events triggered by IP_3 were here recorded as a continuous line, reflecting that catecholamines released from the vesicles after IP_3 stimulation most probably left the cytosol by flowing through the digitonin pores in our recordings. However, their exit through the plasma

Figure 5. The exocytosis evoked by muscarinic stimulation is mediated by extracellular Ca^{2+} together with mobilization of Ca^{2+} from intracellular organelles through an inositol 1,4,5-trisphosphate (IP_3)-mediated process. Cells were voltage clamped, and exocytosis of secretory vesicles was then determined by membrane capacitance (C_m) recordings. Pulses of 5-s duration of 10 μM methacholine (MTC) were applied every 5 min. After responses were stable, different compounds were perfused. *A* and *B*: scheme and protocol of the experiment. 2-APB, 2-aminoethyl diphenylborinate; Baf, bafilomycin A1; CA, catecholamine; Caf, caffeine; MTC, methacholine; Ryn, ryanodine; Thaps, thapsigargin. *C* and *D*: representative C_m recordings of the effect of 100 μM 2-APB (*C*; $n = 12$) and a cocktail of 10 mM Caf, 1 μM Ryn, and 1 μM Thaps (*D*; $n = 17$) on the MTC-mediated exocytosis. Compounds were applied 90 s before the methacholine pulse and then during the rest of the experiment. *E* and *F*: representative C_m recordings of the effect of BAPTA-AM (50 μM) in cells in which this compound increased (*E*; $n = 11$) or decreased (*F*; $n = 6$) the initial exocytotic response. *G*: after MTC elicited stable exocytosis in control conditions, 2 pulses (*1* and *2*) of 2-s Baf (100 nM) were applied before the MTC pulses ($n = 9$). The short application of Baf guaranteed a more selective and less toxic action. *H*: dot plots of the fold times of the response under each condition with respect to their own control. Arrows mean the increase or decrease of the exocytosis exerted by BAPTA-AM. Statistical test: one-sample *t* test (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). *A* created with a licensed version of BioRender.com.

membrane under physiological conditions by means of a nonexocytotic mechanism such as the one reported for amphetamines (21) cannot be discarded. Exocytotic (shown by amperometric spikes; see Refs. 22, 23) and nonexocytotic responses could be recorded in the same cell with different stimuli, such as digitonin or IP_3, respectively (see Fig. 2*D*).

A nonexocytotic release of catecholamines, displayed also as a continuous increase on the baseline, was shown by



Camacho and coworkers (13) using bafilomycin A1 in bovine chromaffin cells due to the alkalinization of the secretory vesicle by blocking the V-ATPase. To investigate whether IP_3 might exhibit a similar mechanism of catecholamine release from secretory vesicles, bafilomycin A1 was tested alone and in combination with IP_3. Also, a plateau-shaped response without additive effects was obtained when both stimuli were applied together, which suggests a similar mechanism of action.

In synaptosome-derived microsomal vesicles, Ca^{2+} acts as coagonist of IP_3 to potentiate Ca^{2+} release from the ER, being required to trigger release (3). In the present study our data show that cytosolic Ca^{2+} is not required to trigger catecholamine release from the vesicle to the cytosol by IP_3 (Fig. 3*C*), although this process is regulated through a bell-shaped cytosolic Ca^{2+}-dependent process. Indeed, catecholamine release was maximal at 300 nM cytosolic Ca^{2+} and then diminished at 1–3 μM Ca^{2+} (Fig. 3*E*). This cytosolic Ca^{2+}-dependent regulation is similar to the Ca^{2+} dependence of the Ca^{2+} release process elicited by IP_3 through ER IP_3Rs (1–3), suggesting that it might derive from the regulation by cytosolic Ca^{2+} of IP_3Rs, most probably vesicular. The biphasic cytosolic Ca^{2+} regulation disappeared in cells in which the intracellular organelles were depleted of free Ca^{2+}, indicating that, in addition, a CICR mechanism might underlie such regulation. Furthermore, other Ca^{2+}-dependent processes such as vesicle transport, docking, and priming might be contributing to the observed bell-shaped secretory response triggered by IP_3.

It is relevant to note that in our present study Ca^{2+} itself triggered catecholamine release in digitonin-permeabilized cells (Fig. 3*F*). Jankowski and colleagues (24) also reported catecholamine release elicited by this cation in bovine chromaffin cells. In our study the release was mainly triggered at 1–3 μM Ca^{2+}, concentrations at which the CICR process takes place. Furthermore, this release elicited by Ca^{2+} before the application of the IP_3 pulse did not occur in thapsigargin-treated cells, further reflecting that it was mediated through a CICR pathway that might contribute to the inhibition of IP_3Rs by high Ca^{2+}. However, this release due to cytosolic Ca^{2+} was not relevant in relation to the total release of catecholamines elicited by IP_3 at concentrations above 100 nM cytosolic Ca^{2+}, since there were no significant statistical differences between control and Ca^{2+}-depleted cells.

A remaining component of catecholamine release persists after the Ca^{2+} depletion procedure in the absence of Ca^{2+} (Fig. 4*E*). Several factors might be contributing to that release. First, it could be affected by bound vesicular Ca^{2+} (11) as occurs in the ER, where Ca^{2+} luminal regulation is mediated through the protein ERp44, which inhibits $IP3R_1$ at Ca^{2+} concentrations below 10 μM (25). In chromaffin vesicles, the interaction of IP_3Rs with ERp44 might be replaced by the interaction with chromogranin B (26), giving rise to a different response in the presence of a certain amount of cytosolic Ca^{2+}. In addition, the activation/inhibition curves of IP_3Rs elicited by cytosolic Ca^{2+} also depend on other several factors such as the IP_3R subtype, the ATP, or the IP_3 concentrations. In this last regard, at low IP_3 values the curve is shifted to the left, and therefore the activation/inhibition occurs at lower Ca^{2+} concentrations (27). Indeed, when pulses of methacholine of different duration were tested, secretory responses could be large or null in the same cell, and they could be inhibited or increased by KPF, respectively (Fig. 6, *E* and *F*). This reflects that the different secretory responses elicited by methacholine could be also due to a different IP_3 concentration induced by the muscarinic agonist, which would lead to a different Ca^{2+} concentration around IP_3Rs, giving as a result "low" or "high" amounts of initial exocytosis, which can be increased or inhibited by BAPTA-AM or KPF depending on the degree of inhibition of the IP_3Rs. The experiments with KPF suggest that exocytosis elicited by muscarinic stimulation is controlled by mitochondria acting as a Ca^{2+} buffer (Fig. 6) and further support the finding of the biphasic Ca^{2+} regulation of the IP_3-triggered exocytotic response. The feedback Ca^{2+} regulation of IP_3Rs by mitochondria has been functionally evidenced in the ER by activation of the MCU with KPF, or by inhibition of the mitochondrial Na^+/Ca^{2+} exchanger with CGP (19, 28–30).

Vesicular Ca^{2+} also has a role in the regulation of the IP_3-triggered response. Bafilomycin A1 abolished the C_m increments elicited by methacholine (Fig. 5*G*). This could be due to Ca^{2+} released from secretory vesicles, which increased the Ca^{2+} concentration around IP_3Rs to values that would inhibit those receptors (1 μM). Indeed, in bovine chromaffin cells, bafilomycin A1 was found to release 2.5% of the total intravesicular Ca^{2+} in Oregon Green 488 BAPTA-2-treated chromaffin vesicles (15). This represents a 1.25 μM Ca^{2+} concentration if 50 μM is taken as the total free Ca^{2+} concentration inside the vesicle.

Conclusions

The main findings of the present study are *1*) IP_3 triggers the release of catecholamines from secretory vesicles to the cytosol, the V-ATPase blockade being a possible mechanism; *2*) this process can occur in the absence of cytosolic Ca^{2+}; *3*) Ca^{2+} from intracellular organelles contributes to vesicular catecholamine release in the absence of cytosolic Ca^{2+} or at basal cytosolic Ca^{2+} concentrations; *4*) cytosolic Ca^{2+} regulates biphasically the IP_3-mediated catecholamine response through a CICR pathway; *5*) Ca^{2+} itself also triggers vesicular catecholamine release, through a CICR mechanism; 6) IP_3 triggers exocytosis of secretory vesicles that also exhibits cytosolic Ca^{2+} regulation; *7*) IP_3-triggered exocytosis might be also regulated by vesicular Ca^{2+}; and *8*) mitochondria

Figure 6. Mitochondria contribute physiologically to the biphasic regulation by cytosolic Ca^{2+} of the inositol 1,4,5-trisphosphate (IP_3)-triggered exocytotic response. Pulses of 5-s duration of 10 μM methacholine (MTC) were applied every 5 min. Cells were voltage clamped, and exocytosis of secretory vesicles was determined by membrane capacitance (C_m) recordings. *A* and *B*: scheme and protocol of the experiment, respectively. CA, catecholamine; KPF, kaempferol. *C*–*F*: representative C_m recordings of the effect of 10 μM KPF applied 90 s before the methacholine pulse and then during the rest of the experiment in cells in which this compound increased (*C*; $n = 13$) or decreased (*D*; $n = 11$) exocytosis, elicited by 5-s pulses of MTC in different cells, or in the same cell elicited by 50-ms (*E*) or 5-s (*F*) pulses of MTC ($n = 3$). *G*: dot plots of the fold times of each condition with respect to their own control. Statistical test: one-sample *t* test (***$P < 0.001$, ****$P < 0.0001$). *A* created with a licensed version of BioRender.com.

regulate the IP_3-elicited exocytosis by taking up cytosolic Ca^{2+}.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by and performed in accordance with the Ethics Committee of the Universidad Autónoma de Madrid and Universidad de Alcalá regulations and conducted according to European Directive 2010/63/EU and Royal Decree 53/2013 from Spain.

DATA AVAILABILITY

Data will be made available upon reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.A. conceived and designed research; S.S.-L., A.J.-P., A.H.-V., B.C.-H., N.G.-M., A.P.-A., J.C.C.-G., and L.R.-R. performed experiments; S.S.-L., A.J.-P., A.H.-V., B.C.-H., N.G.-M., and J.C.C.-G. analyzed data; A.A. interpreted results of experiments; S.S.-L. and A.J.-P. prepared figures; A.A. drafted manuscript; A.A. edited and revised manuscript; S.S.-L., A.J.-P., A.H.-V., B.C.-H., N.G.-M., A.P.-A., J.C.C.-G., L.R.-R., and A.A. approved final version of manuscript.

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