

# Regulation by L channels of $\text{Ca}^{2+}$ -evoked secretory responses in ouabain-treated chromaffin cells

Ricardo De Pascual<sup>1,2</sup> · Inés Colmena<sup>1,2</sup> · Lucía Ruiz-Pascual<sup>1</sup> ·  
Andrés Mateo Baraibar<sup>1</sup> · Javier Egea<sup>1</sup> · Luis Gandía<sup>1,2</sup> · Antonio G. García<sup>1,2,3</sup>

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**Abstract** It is known that the sustained depolarisation of adrenal medullary bovine chromaffin cells (BCCs) with high  $\text{K}^+$  concentrations produces an initial sharp catecholamine release that subsequently fades off in spite depolarisation persists. Here, we have recreated a sustained depolarisation condition of BCCs by treating them with the  $\text{Na}^+/\text{K}^+$  ATPase blocker ouabain; in doing so, we searched experimental conditions that permitted the development of a sustained long-term catecholamine release response that could be relevant during prolonged stress. BCCs were perfused with nominal  $0\text{Ca}^{2+}$  solution, and secretion responses were elicited by intermittent application of short  $2\text{Ca}^{2+}$  pulses (Krebs-HEPES containing  $2\text{ mM Ca}^{2+}$ ). These pulses elicited a biphasic secretory pattern with an initial 30-min period with secretory responses of increasing amplitude and a second 30-min period with steady-state, non-inactivating responses. The initial phase was not due to gradual depolarisation neither to gradual increases of the cytosolic calcium transients ( $[\text{Ca}^{2+}]_c$ ) elicited by  $2\text{Ca}^{2+}$  pulses in BCCs exposed to ouabain; both parameters increased soon after ouabain addition. Nifedipine blocked these responses, and FPL64176 potentiated them, suggesting that they were triggered by  $\text{Ca}^{2+}$  entry through non-inactivating

L-type calcium channels. This was corroborated by nifedipine-evoked blockade of the L-type  $\text{Ca}^{2+}$  channel current and the  $[\text{Ca}^{2+}]_c$  transients elicited by  $2\text{Ca}^{2+}$  pulses. Furthermore, the plasmalemmal  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) blocker SEA0400 caused a mild inhibition followed by a large rebound increase of the steady-state secretory responses. We conclude that these two phases of secretion are mostly contributed by  $\text{Ca}^{2+}$  entry through L calcium channels, with a minor contribution of  $\text{Ca}^{2+}$  entry through the reverse mode of the NCX.

**Keywords** Ouabain · Chromaffin cell · L-type  $\text{Ca}^{2+}$  channels · N-type  $\text{Ca}^{2+}$  channels · P/Q-type  $\text{Ca}^{2+}$  channels · NCX · Catecholamine release · Amperometry

## Introduction

In a classical experiment done at the perfused bovine adrenal gland, Baker and Rink [7] found that the sustained depolarisation of medullary chromaffin cells with a high potassium solution ( $\text{K}^+$ ) induced a sharp catecholamine release response that rapidly faded in spite of the fact that cell depolarisation persisted. The authors attributed such secretion decay to a probable inactivation of an as yet uncharacterised voltage-activated calcium channel current ( $I_{\text{Ca}}$ ). The whole cell inward  $I_{\text{Ca}}$  of bovine chromaffin cells (BCCs) was first characterised at Erwin Neher's laboratory [18]. By using selective blockers of calcium channel subtypes, we later on found that such current was contributed by three of the subtypes of voltage-activated calcium channels (VACCs) described in neurons [44], namely, 20 % L-type ( $\alpha_{1D}$ , Cav 1.3), 30 % N-type ( $\alpha_{1B}$ , Cav 2.2) and 50 % PQ-type ( $\alpha_{1A}$ , Cav 2.1) [2, 3, 20, 22, 50].

Ricardo De Pascual and Inés Colmena are equal contributors.

✉ Antonio G. García  
agg@uam.es

<sup>1</sup> Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4, 28029 Madrid, Spain

<sup>2</sup> Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, C/ Arzobispo Morcillo, 4, 28029 Madrid, Spain

<sup>3</sup> Servicio de Farmacología Clínica, Instituto de Investigación Sanitaria, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, c/ Diego de León, 62, 28006 Madrid, Spain

Three different VACCs expressed within the same chromaffin cell raise the relevant physiological question on whether they play different roles in controlling various functions, particularly the calcium-dependent exocytotic release of catecholamine and the calcium-dependent endocytotic process [23, 37]. The following findings support some specific characteristics and different functions for channel subtypes [37]: (i) N and PQ currents are regulated by neurotransmitters in a voltage-dependent manner while L current regulation is voltage-independent [1, 21, 28]; (ii) depending on the experimental conditions, exocytosis triggered by  $K^+$  pulses may be preferentially coupled to L channels [36] or to PQ channels [33]; (iii) secretion triggered by trains of action potentials is controlled by PQ channels while with depolarising pulses, other channels participate [12]; (iv) the calcium-dependent endocytosis is mostly controlled by L channels [49, 51]; (v) N and PQ channels undergo a pronounced voltage-dependent inactivation while L channels are resistant to such inactivation [29, 56]; (vi) L channels undergo calcium-dependent inhibition at a rate slower than N and PQ channels [30]; (vii) calcium entering the cell through the lesser inactivating L channels serves to modulate the more inactivating N and PQ channels [50].

The simple picture on the role of calcium in the stimulus-secretion coupling process [14] was based in the pioneering experiment of Douglas and Rubin [16] in the perfused cat adrenal gland: the acetylcholine-triggered secretion required external  $Ca^{2+}$  entry through a  $Ca^{2+}$ -permeable membrane pathway [15]. However, we now know that  $Ca^{2+}$  ions intervene not only in the final steps of exocytosis but also at pre-exocytotic steps. In this context, the early supposition of Baker and Rink [7] in the sense that the transient secretory response triggered by sustained  $K^+$  depolarisation of BCCs undergo inactivation, could certainly find a partial explanation in the voltage- and calcium-dependent inactivation of VACCs and of  $Ca^{2+}$  entry, as suggested by these authors. However, in the context of new knowledge accumulated since 1975, the inactivation of secretion occurring in spite of sustained depolarisation may be better explained by including additional factors. For instance, not all channel subtypes inactivate similarly, as discussed above. Thus, in perfused BCCs, we found that sustained  $K^+$  depolarisation caused a secretory response with two components. One accounted for 70–80 % of the secretory response and was blocked by  $\omega$ -conotoxin GVIA (GVIA) and  $\omega$ -agatoxin IVA (IVA) and by sustained depolarisation; therefore, it was linked to  $Ca^{2+}$  entry mostly through N and PQ channels. The other component was sensitive to 1–4-dihydropyridines and was resistant to inactivation, generating a low rate but sustained secretion [57]. Thus, it seems that sustained  $K^+$  depolarisation triggers a secretory response in BCCs with a fast initial peak component associated to inactivating N and PQ channels that fades off to reach a lower secretion steady-state plateau, sustained by  $Ca^{2+}$  entry through non-inactivating L channels.

The present study focuses on this problem with two major new experimental approaches: (1) instead of  $K^+$ , the cardiac glycoside ouabain was used to elicit depolarisation of BCCs through the inhibition of the membrane  $Na^+/K^+$ -ATPase (NKA) [6]; and (2) instead of room temperature, the secretion experiments were performed at 37 °C because at this physiological temperature, higher rates of exocytosis have been reported to occur in BCCs [9, 25, 27, 32, 46, 59]. We here report that in BCCs perfused in a  $0Ca^{2+}$  solution containing ouabain, the intermittent application of 2 mM  $Ca^{2+}$  pulses caused increasing secretory responses that reached a steady-state, non-inactivating plateau in about 30 min. Such responses were mostly due to  $Ca^{2+}$  entry through L channels although a minor contribution of the plasmalemmal  $Na^+/Ca^{2+}$  exchanger (NCX) was also present. Considering that an endogenous ouabain-like steroid is known to be synthesised into and released from the bovine adrenal cortex [31, 52], our present results may have further physiological relevance in the sense that endogenous ouabain could somehow contribute to the maintenance of a long-lasting catecholamine release response during prolonged stressful conflicts.

## Materials and methods

### Isolation and culture of bovine chromaffin cells

All experiments were carried out in accordance with the guidelines established by National Council on Animal Care and were approved by the local Animal Care Committee of the Universidad Autónoma de Madrid. Bovine adrenal glands were obtained from a local slaughterhouse under the supervision of the local veterinary service.

Chromaffin cells were isolated following standard methods [35] with some modifications [41]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % foetal calf serum, 10  $\mu$ M cytosine arabinoside, 10  $\mu$ M fluorodeoxyuridine, 50 IU  $mL^{-1}$  penicillin and 50  $\mu$ g  $mL^{-1}$  streptomycin. For catecholamine secretion experiments, cells were plated on plastic Petri dishes (60 mm) at a density of  $10^6$  cells  $mL^{-1}$  (5 mL per dish). For measurements of ionic currents with the patch-clamp technique, cells were plated on 1-cm diameter glass coverslips at a density of  $5 \times 10^4$  cells/coverslip. Cells were kept for 1–4 days at 37 °C in a water-saturated incubator, with a 5 %  $CO_2/95$  % air atmosphere. After 24 h, the medium was replaced by serum-free fresh medium, and subsequently changed every 2 days.

### On-line measurement of catecholamine release from intact bovine chromaffin cell populations

Cells were scrapped off carefully from the bottom of the Petri dish with a rubber policeman and centrifuged at 800 rpm for

10 min. The cell pellet was resuspended in 200  $\mu\text{L}$  of Krebs-HEPES (composition in mM: NaCl 144; KCl 5.9;  $\text{MgCl}_2$  1.2; glucose 11; HEPES 10; pH 7.4); the concentration of  $\text{CaCl}_2$  varied depending on the protocol. Cells were introduced in a microchamber for their superfusion at the rate of  $2 \text{ mL min}^{-1}$ .

The microchamber had a volume of 100  $\mu\text{L}$  and was covered with a jacket for the continuous external circulation of water at  $37^\circ\text{C}$ . For the detection of released catecholamines, the liquid flowing from the superfusion chamber reached an electrochemical detector (model Metrohn AG CH-9100 Herisau, Switzerland) equipped with a glassy carbon working electrode Ag/AgCl reference electrode and a gold auxiliary electrode. Catecholamines were oxidised at  $+0.65 \text{ V}$ , and the oxidation current was recorded on a PC computer placed just at the outlet of the microchamber, which monitors on line, under the amperometric mode, the amount of catecholamines secreted [11]. Cells were stimulated to secrete with 10-s pulses. Solutions were rapidly exchanged through electrovalves commanded by a computer.

### Recording of calcium channel currents

Membrane currents were measured using the whole cell configuration of the patch-clamp technique [26]. Coverslips containing the cells were placed in an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused with a control Tyrode solution containing (in mM): NaCl 137,  $\text{MgCl}_2$  1,  $\text{CaCl}_2$  2, HEPES/NaOH 10, pH 7.4. For current recordings,  $2 \text{ mM Ca}^{2+}$  was used as charge carrier. The cells were internally dialysed with a solution containing (in mM): NaCl 10, CsCl 100, tetraethylammonium.Cl 20, MgATP 5, 3-ethyleneglycol-bis-( $\alpha$ -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 14, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES)/CsOH 20, Na.GTP 0.3, pH 7.2.

Whole cell recordings were made with fire-polished glass electrodes (resistance 2–5  $\text{M}\Omega$ ) mounted on the headstage of an EPC-9 patch-clamp amplifier, allowing cancellation of capacitive transients and compensation of series resistance. All recordings and data analysis were carried out with the software PULSE (HEKA Elektronik, Lambrecht, Germany). Unless indicated otherwise, the cells were clamped at  $-80 \text{ mV}$  holding potential. Step depolarisations to 0–10 mV from this holding potential lasted 50 ms and were applied at 30-s intervals, to minimise current rundown [19]. Cells with pronounced rundowns were discarded.

External solutions were exchanged by a fast superfusion device consisting of a modified multi-barrelled pipette, the common outlet of which was positioned 50–100  $\mu\text{m}$  from the cell. Control and test solutions were changed using miniature solenoid valves operated manually (The Lee Company, Westbrook, CT, USA). The flow rate ( $0.5\text{--}1 \text{ mL min}^{-1}$ ) was

regulated by gravity to achieve a complete replacement of cell surroundings in less than 1 s.

### Recording of membrane potential and action potentials

Recordings of membrane potential ( $V_m$ ) and APs were made under the current-clamp mode in the whole cell configuration of the patch-clamp technique [26], which allows the observation of spontaneous variations in the  $V_m$ .

Cells were superfused with control Tyrode solution at pH 7.4 containing (in mM): 137 NaCl, 1  $\text{MgCl}_2$  and 10 HEPES/NaOH; an intracellular solution at pH 7.4 containing (in mM) 135 KCl, 10 NaCl, 10 HEPES, 1  $\text{MgCl}_2$  and 5 EGTA was introduced in the patch-clamp pipette.

### Measurement of cytosolic calcium concentrations

The changes of the cytosolic calcium concentrations ( $[\text{Ca}^{2+}]_c$ ) triggered by  $\text{Ca}^{2+}$  pulses were monitored using the fluorescent  $\text{Ca}^{2+}$  probe fluo-4. Two-day post-plated cells at a density of  $2 \times 10^5$  cells per well on 25-mm diameter Petri dishes were loaded with 3  $\mu\text{M}$  fluo-4/AM for 45 min at  $37^\circ\text{C}$  in Krebs-HEPES solution containing the following composition (in mM): 140 NaCl, 5.6 KCl, 1.2  $\text{MgCl}_2$ , 2  $\text{CaCl}_2$ , 10 HEPES, 11 D-glucose, pH 7.4. Loading with fluorescent dye was terminated by washing the Petri dishes, containing the attached cells, twice with Krebs-HEPES; then, cells were kept at room temperature for 15 min before placing them in the head stage of a Nikon eclipse TE-300 microscope equipped with shutter lambda 10–2 filter wheels instrument (Shutter instruments Co. Novato, CA, USA) to measure their fluorescence. Fluo-4 was excited with light at 485 nm. Emitted light was transmitted through a 505-nm dichroic mirror and a 520-nm barrier filter before being detected by a Hamamatsu cooled CCD-camera Orca-G (Hamamatsu Photonics Inc., Higashi-Ku, Hamamatsu City, Japan). Data were acquired and analysed using the Metafluor v 2.2 Imaging System (Molecular Devices).

### Materials and solutions

The following materials were used: collagenase type I from Sigma (Madrid, Spain); Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin fraction V, foetal calf serum, and antibiotics were from Gibco (Madrid, Spain). All other chemicals used were reagent grade from Merck and Panreac Química (Madrid, Spain). Agatoxin-IVA was from PeptaNova GmbH (Sandhausen, Germany), and  $\omega$ -conotoxin GVIA was from Bachem Feinchemikalien (Budendorf, Switzerland). Agatoxin-IVA and  $\omega$ -conotoxin GVIA were dissolved in distilled water and stored frozen in aliquots at 0.1 mM. Nifedipine ( $10^{-2} \text{ M}$ ) was prepared in dimethylsulphoxide (DMSO) and protected from light. Final concentrations of drugs were obtained by diluting the stock solutions directly

into the extracellular solution. At these dilutions, solvents had no effect on the parameters studied.

## Statistics

The results are presented as means  $\pm$  SEM. The measurements of peak current and charge that flow through the channels after their activation were calculated with the programme PULSE.  $IC_{50}$  values were estimated through non-linear regression analysis using OriginPRO software (OriginLab Corporation, Northampton, USA). Data analysis of amperometric recording in BCC populations (peak amplitudes in nA or areas in pC) was performed using OriginPro software. Data from measurement of changes in the  $[Ca^{2+}]_c$  were acquired and analysed using the Metafluor v2.2 Imaging System (Molecular Devices, Sunnyvale, CA 94089 USA). Graphs and the mathematical analyses were performed using the GraphPad Prism software, version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between means of group data were performed by one-way analysis of variance (ANOVA) followed by the Duncan post hoc test when appropriate.

## Results

This study was aimed at exploring the secretory responses elicited by  $Ca^{2+}$  pulses and the contribution of plasmalemmal VACC subtypes and the NCX to those responses, in BCCs chronically exposed to 10  $\mu$ M ouabain to block the sodium pump at 37 °C. This physiological temperature was selected because the secretory responses of chromaffin cells are substantially higher at 37 °C, compared with room temperature used by most researchers in this field. Thus in spite of the fact that temperature does not affect  $I_{Ca}$  currents in BCCs, the  $K^+$  secretory responses are 3-fold higher at 37 °C, with respect to 22 °C [46]. The secretion experiments here presented were done in BCCs that were continuously fast-perifused with Krebs-HEPES at 37 °C, containing nominal  $0Ca^{2+}$  in the presence of 10  $\mu$ M ouabain. This ouabain concentration was chosen on the basis of previous experiments showing that it caused near full inhibition of the NKA in BCCs [6]. The  $0Ca^{2+}$  solution prevented the development of an increased basal secretion known to occur in incubated ouabain-treated BCCs [6].

### Secretory responses triggered by repeated calcium pulses: effects of ouabain, nifedipine, $\omega$ -toxins and FPL64176

In a classical experiment from the laboratory of William Douglas, the  $Ca^{2+}$  pulse protocol was used to demonstrate that after a period of perfusion with saline deprived of divalent cations (i.e.  $Mg^{2+}$  and  $Ca^{2+}$ ), the introduction of  $Ca^{2+}$

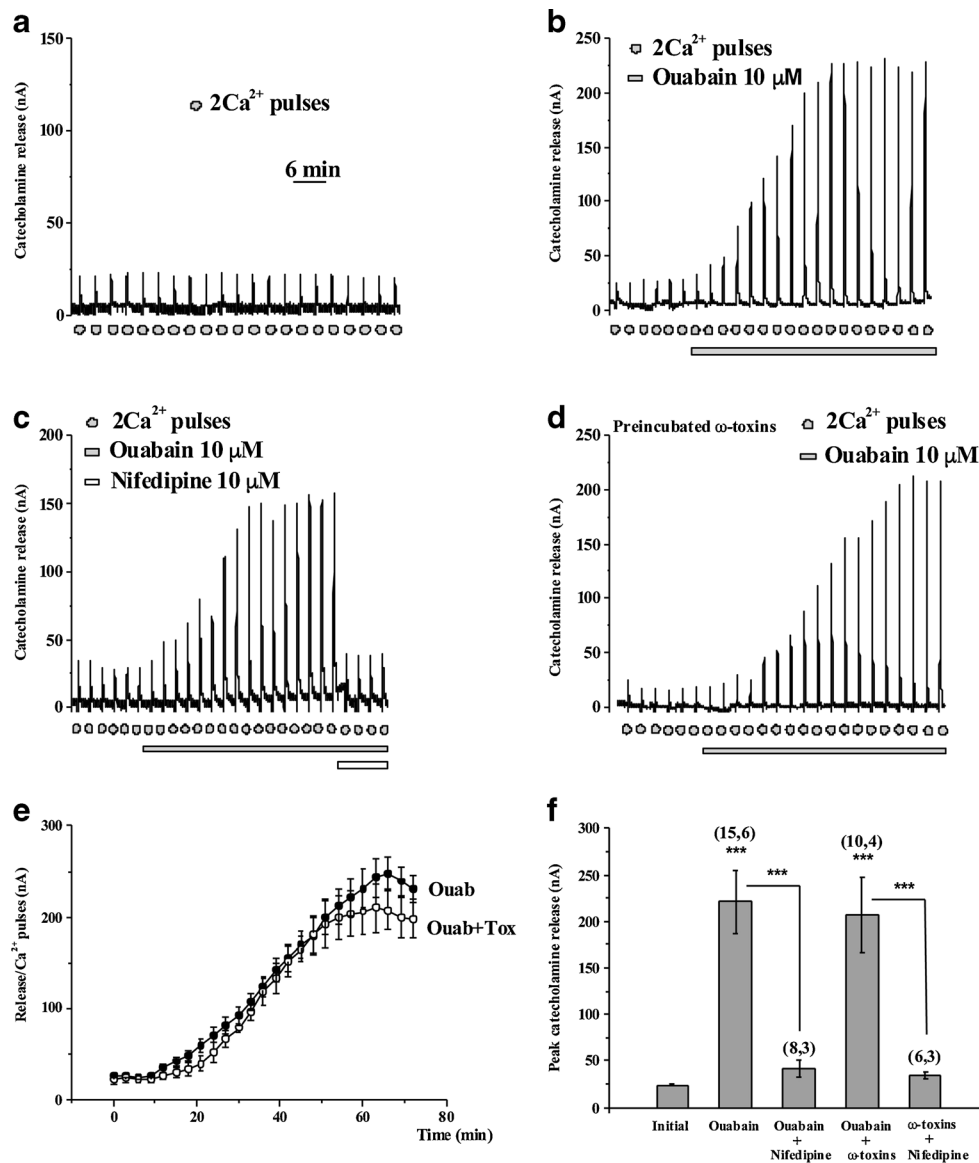
triggered a healthy catecholamine release response in the perfused cat adrenal gland [17]. In the case of BCCs (5 millions trapped in a microchamber) that were being perfused in nominal  $0Ca^{2+}$  (in the presence of  $Mg^{2+}$  at 1.2 mM), the repeated applications of 2 mM  $Ca^{2+}$  pulses for 10 s at 3-min intervals produced a tiny secretion that was maintained at around 20 nA along 1 h and 21 sequential  $2Ca^{2+}$  pulses (Fig. 1a). Those responses could be generated by tiny  $Ca^{2+}$  entry through store-operated  $Ca^{2+}$  channels (SOC) that could be activated by partial depletion of the endoplasmic reticulum (ER)  $Ca^{2+}$  store in cells that were being perfused with  $0Ca^{2+}$  during the  $2Ca^{2+}$ -pulse intervals. It could also be due to  $Ca^{2+}$  entry through the reverse mode of the plasmalemmal NCX.

In a similar experiment and after the initial application of six sequential  $2Ca^{2+}$  pulses, ouabain at 10  $\mu$ M was perfused; the secretory responses began to increase in amplitude to reach a plateau at around 200 nA after giving 11 additional  $2Ca^{2+}$  pulses (30 min in the presence of ouabain). In the last 6–7 pulses, still in the presence of ouabain, the secretion responses reached a steady-state plateau (Fig. 1b).

Experiments to test the contribution of VACC subtypes were next performed using selective blockers of L channels (10  $\mu$ M nifedipine) or combined blockers of N channels (1  $\mu$ M GVIA) and PQ channels (3  $\mu$ M IVA). In the experiment of Fig. 1c, once the secretory responses stabilised at around 150 nA, the introduction of nifedipine on top of ouabain drastically reduced the response to near pre-ouabain levels (Fig. 1c).

In the experiment of Fig. 1d, the BCCs were preincubated with the  $\omega$ -toxins for 45 min because their expensiveness precluded their continuous application in the medium that perfused the cells. These toxins are known to irreversibly bind to N and PQ channels [3, 30], and thus, we assumed that in the present experiments, they are blocking the N and PQ channels. In spite of this channel blockade, in the  $\omega$ -conotoxin-treated BCCs, the  $Ca^{2+}$  pulses still elicited large secretory responses at around 200 nA, similar to those of previous experiments (Fig. 1d). In an additional experiment, the  $\omega$ -conotoxin-preincubated cells were challenged with  $2Ca^{2+}$  pulses, and once the steady state of secretion was reached at around 270 nA, nifedipine was added on top of ouabain; once more, nifedipine caused a rapid and drastic blockade of secretion (not shown). It was interesting that nifedipine brought the steady-state response to the initial levels, suggesting that this tiny response was not associated to  $Ca^{2+}$  entry through L-type VACCs; as stated before, such responses could be due to tiny  $Ca^{2+}$  entry through the reverse NCX. The time courses of the averaged secretory responses in cells treated with ouabain alone or  $\omega$ -toxins plus ouabain are plotted in Fig. 1e; they were similar. Averaged data are graphed in Fig. 1f; ouabain augmented secretion at a peak of  $220 \pm 35$  nA, 10-fold higher than the initial secretion. Nifedipine reduced secretion to near initial values both in cells





**Fig. 1** Gradual augmentation of catecholamine release elicited by calcium pulses in BCCs chronically exposed to ouabain, and its blockade by nifedipine but not by  $\omega$ -toxins. BCCs (5 millions), perfused with Krebs-HEPES containing nominal  $0\text{Ca}^{2+}$  at  $37^\circ\text{C}$ , were intermittently challenged with 10-s  $2\text{Ca}^{2+}$  pulses (Krebs-HEPES containing  $2\text{mM Ca}^{2+}$ ) at 3-min intervals, as indicated by dots at the bottom of the original secretion records of panels **a** to **d**. Cells were perfused with  $10\ \mu\text{M}$  ouabain during the time lapse indicated by the *horizontal bars at the bottom*. In the experiment of panel **c**,  $10\ \mu\text{M}$  nifedipine was applied on top of ouabain, as indicated by the *bottom white horizontal bar*. In panel **d**, cells were preincubated at room

temperature with  $1\ \mu\text{M}$   $\omega$ -conotoxin GVIA and  $3\ \mu\text{M}$   $\omega$ -agatoxin IVA during 45 min, before they were collected from the Petri dish and placed in the microchamber to perform the experiment. Panel **e** shows the time course of secretory responses triggered by  $2\text{Ca}^{2+}$  pulses in cells treated with ouabain alone (ouab) or with ouabain plus  $\omega$ -toxins, at 3-min intervals (time in the abscissa); amplitude of secretory spikes in the ordinate (in nA). **f** Pooled averaged data on maximum secretion peaks in the presence of the treatments. The highest peak secretion (in nA of catecholamine oxidation current in the ordinate) was taken in each individual experiment. Data in **f** are means  $\pm$  SEM of the number of experiment (n) and cultures (N) shown in parentheses. \*\*\* $p < 0.001$

treated only with ouabain and in those preincubated with  $\omega$ -toxins and subsequently treated with ouabain.

Similar experiments with L channel activator FPL64176 [39] were performed next. In the experiment displayed in Fig. 2a, ouabain augmented the secretory responses elicited by repeated  $2\text{Ca}^{2+}$  pulses that reached a steady-state peak secretion at around 200 nA. Added on top of ouabain,

FPL64176 at  $0.3\ \mu\text{M}$  caused a notable further increase to a new steady-state level of secretion, at around 400 nA. Finally, when added on top of ouabain and FPL64176, nifedipine at  $10\ \mu\text{M}$  near fully blocked the secretory responses. Pooled data are graphed in Fig. 2b. Ouabain augmented by 10-fold the initial secretion that was further enhanced by 18-fold in the presence of ouabain plus

FPL64176 and was reduced to the initial level of secretion by nifedipine.

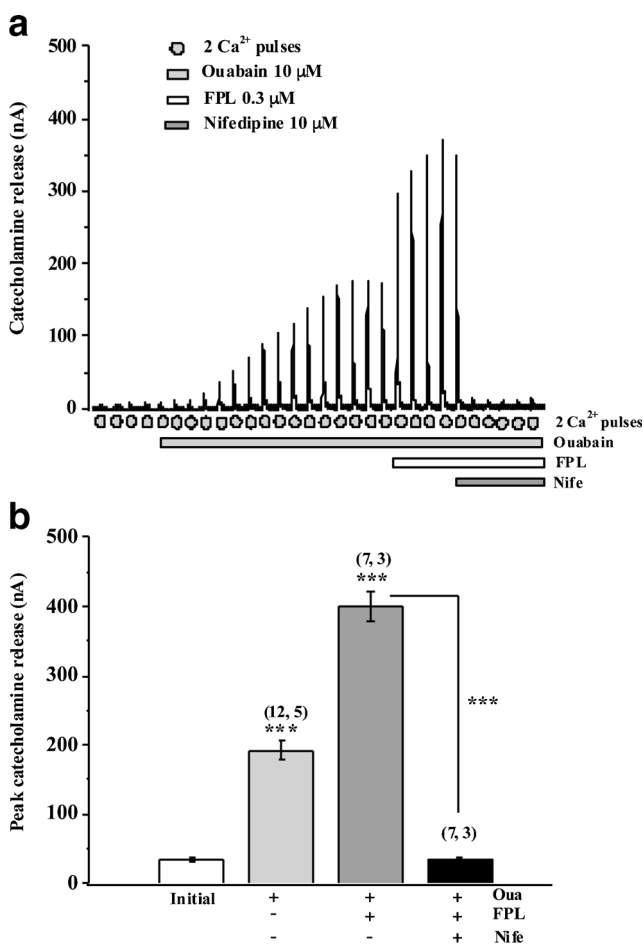
### Secretory responses triggered by repeated potassium pulses: effects of ouabain, nifedipine and $\omega$ -toxins

As the potentiation of secretion was likely due to the indirect cell depolarising effects of ouabain, it was of interest to see whether such potentiation was produced also when the cells were challenged with a  $K^+$  pulse to elicit a direct cell depolarisation. In fact, in cells perfused with a 2 mM  $Ca^{2+}$  solution, we previously found that secretion triggered by 10-s pulses of a Krebs-HEPES solution containing 35 mM  $K^+$  and 2 mM  $Ca^{2+}$  ( $35 K^+/2Ca^{2+}$ ) was gradually enhanced in BCCs treated with ouabain [40]. Thus, we explored here how the  $\omega$ -

toxins and nifedipine affected such responses in cells that were continuously perfused with  $0Ca^{2+}$  and ouabain. The experimental designs were similar to those described in Fig. 1 for the  $2Ca^{2+}$  pulses, and the  $35K^+/2Ca^{2+}$  pulses were applied for 10 s at 3-min intervals.

Figure 3a displays a record from an experiment in which the BCCs were repeatedly challenged with  $35K^+/2Ca^{2+}$  pulses, showing tiny secretory responses of around 20–25 nA. In the experiments of Fig. 3b, the introduction of 10  $\mu$ M ouabain from the 5th  $35K^+/2Ca^{2+}$  pulse onwards gradually augmented the secretory responses that reached a plateau at around 100 nA at the 12th  $35K^+/2Ca^{2+}$  pulse given in the presence of ouabain. In a similar experiment, the introduction of 10  $\mu$ M nifedipine reduced back the augmented secretion responses to pre-ouabain levels (Fig. 3c). Furthermore, in cells preincubated with  $\omega$ -toxins (as in Fig. 1d, e), the augmentation by ouabain of the responses to the  $35K^+/2Ca^{2+}$  pulses was unaffected (Fig. 3d); additionally, in these  $\omega$ -toxin-treated cells, nifedipine still produced a drastic reduction of the  $35K^+/2Ca^{2+}$  responses that had been previously augmented by ouabain (Fig. 3e).

Pooled averaged data from experiments performed with those distinct protocols are summarised in Fig. 3f. Ouabain augmented the  $35K^+/2Ca^{2+}$  secretion response from  $22 \pm 2.5$  to  $180 \pm 12$  nA; nifedipine reduced the response to  $30 \pm 1.6$  nA. In BCCs preincubated with  $\omega$ -toxins, ouabain caused a similar augmentation of secretion ( $194 \pm 16$  nA), and this response was reduced by nifedipine to  $35 \pm 1.2$  nA. In brief, the effects of ouabain, nifedipine and  $\omega$ -toxins seemed to be quite similar with the two patterns of stimulation of the BCCs, namely,  $2Ca^{2+}$  pulses (Fig. 1) and  $35K^+/2Ca^{2+}$  pulses in BCCs continuously perfused with a  $0Ca^{2+}$  solution (Fig. 3).

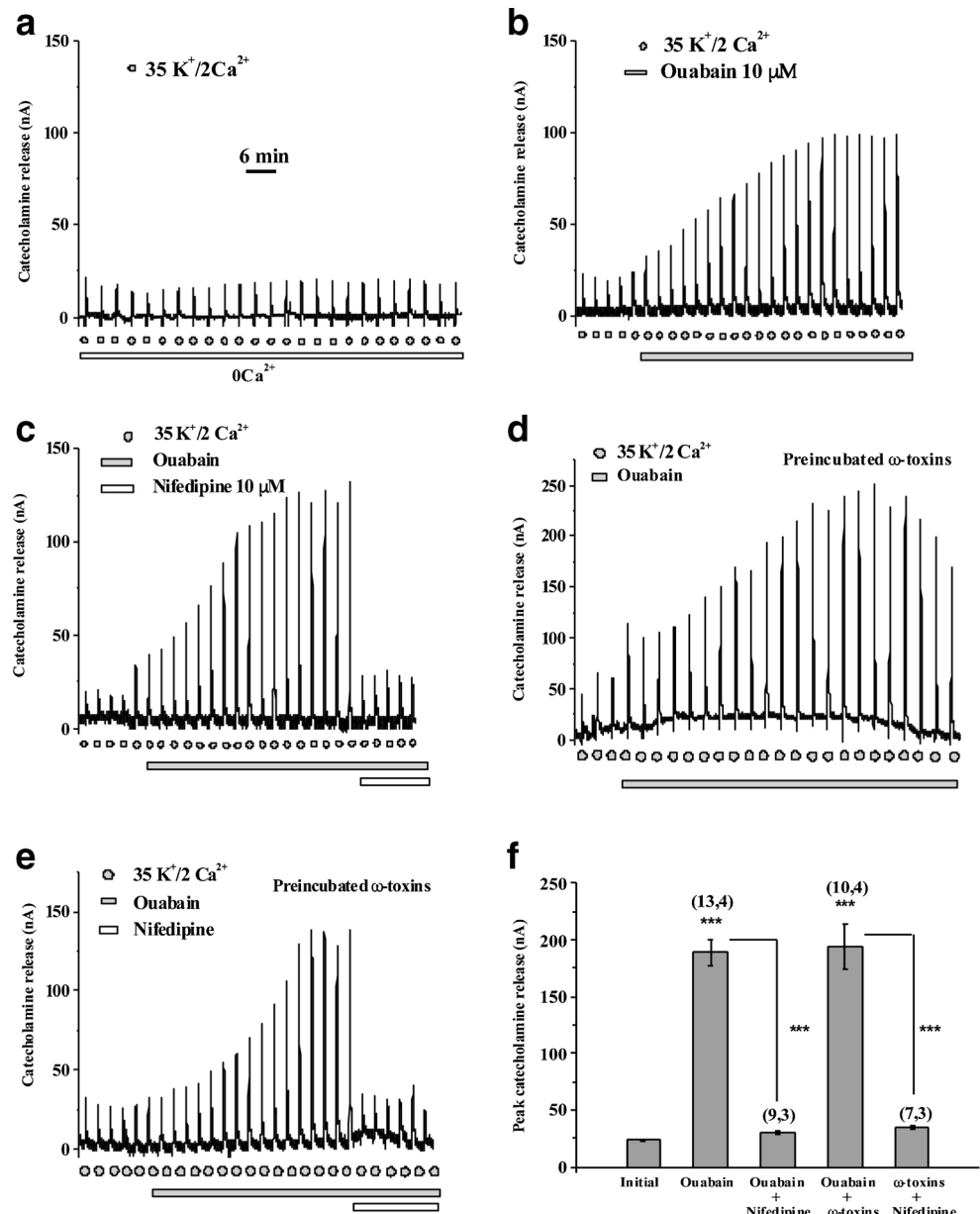


**Fig. 2** Additive augmentation by ouabain and compound FPL64176 of catecholamine release elicited by calcium pulses, and its blockade by nifedipine. BCCs (5 millions) were perfused with  $0Ca^{2+}$  Krebs-HEPES solution and were challenged with 10-s pulses of the  $2Ca^{2+}$  solution (Krebs-HEPES containing 2 mM  $Ca^{2+}$ ), at 37 °C (*bottom dots*). Ouabain (10  $\mu$ M), 0.3  $\mu$ M FPL64176 (FPL), and 10  $\mu$ M nifedipine were introduced as indicated by the *bottom horizontal bars* in panel **a**. **b** Averaged pooled data on peak maximal secretion obtained upon each treatment, normalised in the ordinate as % of initial secretion in each individual experiment. Data are means  $\pm$  SEM of the number of experiments (n) and cultures (N) shown in parenthesis. \*\*\* $p < 0.001$

### Secretory responses triggered by calcium pulses: effects of compounds KB-R7943 and SEA4000

As mentioned in the “Introduction”, the inhibition of NKA by ouabain increases  $[Na^+]_c$  and activates the reverse mode of the NCX (rNCX) [10]. KBR [60] has been widely used to block the NCX; however, this compound has poor selectivity and targets other ion channels and transporters, i.e. the native nicotinic receptors  $\alpha3\beta4$  of BCCs as well as the  $\alpha3\beta4$  and  $\alpha7$  nicotinic receptors expressed in oocytes [48]. Later on, the more selective and potent NCX blocker SEA appeared [38]. Thus, we felt of interest to test the effects of these two NCX blockers on the secretion elicited by  $2Ca^{2+}$  pulses in ouabain-treated BCCs perfused with  $0Ca^{2+}$  at 37 °C. It is known that in chromaffin cells, the  $Na^+/Ca^{2+}$  co-transport through the NCX is 5-fold higher at 37 °C with respect to room temperature [47, 55].

**Fig. 3** Gradual augmentation by ouabain of catecholamine release elicited by potassium pulses and its blockade by nifedipine. BCCs perfused with Krebs-HEPES containing nominal  $0\text{Ca}^{2+}$  at  $37^\circ\text{C}$  were intermittently challenged with 10-s  $35\text{K}^+/2\text{Ca}^{2+}$  at 3-min intervals, as indicated by dots at the bottom of the original secretion records of panels a to e. Cells were perfused with  $10\ \mu\text{M}$  ouabain during the time period indicated by the horizontal bars at the bottom. In panels c and e,  $10\ \mu\text{M}$  nifedipine was applied on top of ouabain, as indicated by the bottom white horizontal bars. In panels d and e, cells were preincubated at room temperature with  $1\ \mu\text{M}$   $\omega$ -conotoxin GVIA and  $3\ \mu\text{M}$   $\omega$ -agatoxin IVA during 45 min, before they were collected from the Petri dish and placed in the microchamber to perform the experiment. f Pooled averaged data of the experiments done in cells that were treated as indicated in panels a to e. The highest peak secretion (in nA of catecholamine oxidation current in the ordinate) was taken in each individual experiment. Data in f are means  $\pm$  SEM of the number of experiments (n) and cultures (N) shown in parenthesis. \*\*\* $p < 0.001$

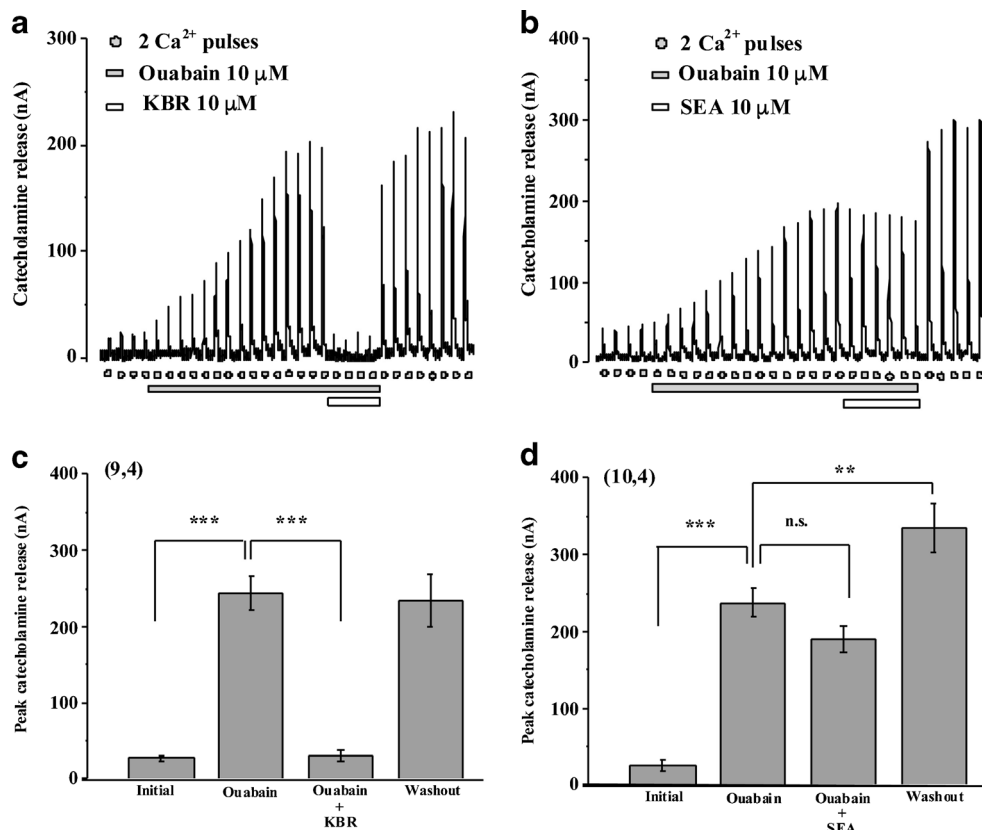


In the experiments of Fig. 4a, the initial secretion elicited by  $2\text{Ca}^{2+}$  pulses of about 20–25 nA was gradually enhanced by  $10\ \mu\text{M}$  ouabain to reach a plateau of around 230 nA. Added on top of ouabain, KBR at  $10\ \mu\text{M}$  drastically reduced the responses to the initial level; upon KBR washout, the secretory response recovered the ouabain-plateau levels. A similar experiment was performed with SEA (Fig. 4b). Once more, ouabain caused a gradual increase of the secretory response elicited by the  $2\text{Ca}^{2+}$  pulses that reached a plateau at around 220 nA. Added on top of ouabain, SEA at  $10\ \mu\text{M}$  elicited a mild decrease of secretion (from 220 to 200 nA); surprisingly and opposite to KBR, after SEA washout, a pronounced rebound increase of secretion occurred. In these

experiments, ouabain was removed from the perfusion fluid together with the NCX blocker; however, it is well established that the ouabain effects on secretion remain for a long time after its washout [24].

Pooled data graphed in Fig. 4c indicate that the initial secretion elicited by  $2\text{Ca}^{2+}$  pulses augmented to  $245 \pm 22$  nA upon ouabain treatment (10-fold increase). Added on top of ouabain, KBR drastically reduced the response to  $30 \pm 7$  nA. This response recovered the previous ouabain peak upon KBR washout. In the case of SEA, it was noteworthy that the peak ouabain response of  $237 \pm 18$  nA was reduced to  $189 \pm 17$  nA, but this effect was not statistically significant. Upon SEA washout, however, secretion reached a peak of  $335 \pm 32$  nA, a significant increase.

**Fig. 4** Effects of blockers of the plasmalemma  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX), KBR and SEA, on secretion triggered by calcium pulses. In panels **a** and **b**, BCCs were perfused at 37 °C with a nominal  $0\text{Ca}^{2+}$  Krebs-HEPES and intermittently challenged with 10-s pulses of a solution containing 2 mM  $\text{Ca}^{2+}$  ( $2\text{Ca}^{2+}$  pulses) applied at 3-min intervals (*bottom dots*). Ouabain at 10  $\mu\text{M}$ , KBR at 10  $\mu\text{M}$  (panel **a**), and SEA at 10  $\mu\text{M}$  (panel **b**) were applied during the time periods indicated by the *bottom horizontal bars*. The ordinates are the amplitudes of the secretory responses in nA of oxidised catecholamine. Panels **c** and **d** are the pooled data of experiments repeated following the protocols of those displayed in panels **a** and **b**, respectively. They are means  $\pm$  SEM of the number of experiments (n) and cultures (N) shown in parenthesis.  $^{***}p < 0.01$ ,  $^{***}p < 0.001$



### Effects of compound KB-R7943 and SEA0400 on sodium and calcium currents

Because of the above-mentioned limited selectivity of KBR to block the NCX, it was convenient to study its ability to block sodium and calcium currents ( $I_{\text{Na}}$ ,  $I_{\text{Ca}}$ ) in voltage-clamped cells. Inward whole cell currents were generated by 50-ms test pulses to 0 mV, applied from a holding potential of  $-80$  mV. The first fast inactivating component of the current was due to  $\text{Na}^+$  entry ( $I_{\text{Na}}$ ), and the second noninactivating component was due to  $\text{Ca}^{2+}$  entry ( $I_{\text{Ca}}$ ) (Figs. 5a, c, arrows).

In the experiment of Fig. 5a, after current stabilisation with repeated test pulses, the successive application of 1 and 10  $\mu\text{M}$  KBR caused a tiny reduction of  $I_{\text{Na}}$  and  $I_{\text{Ca}}$ . On quantitative terms, such reduction accounted for 20 % for  $I_{\text{Na}}$  and 30 % for  $I_{\text{Ca}}$  at 10  $\mu\text{M}$  of KBR (Fig. 5b). Given that in BCCs, the L channel component of  $I_{\text{Ca}}$  accounts for about 20 %, and the fact that KBR near fully blocked the secretion responses elicited by  $2\text{Ca}^{2+}$  pulses, it seems reasonable to suspect that at 10  $\mu\text{M}$ , KBR is selectively blocking the L-type VACCs of BCCs. This was corroborated by occlusion experiments done with nifedipine and KBR. We found that nifedipine occluded the blocking effect of KBR and that KBR occluded the blocking effect of nifedipine (Fig. 6).

Figure 6a shows the time course of  $I_{\text{Ca}}$  (ordinate in pA) and the time-dependent blockade by about 20–25 % elicited by

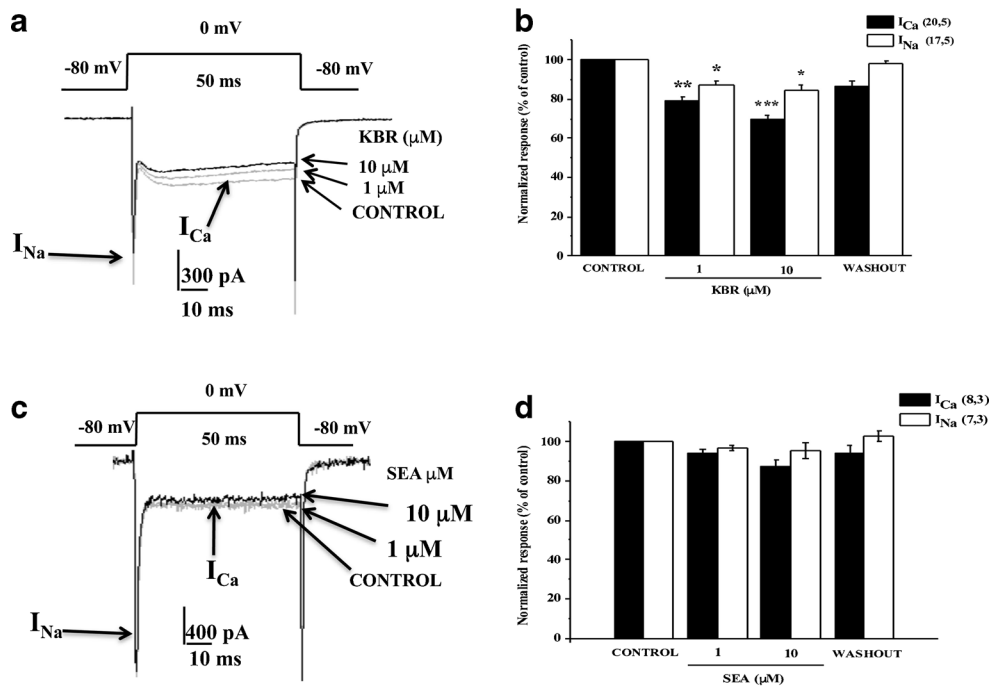
10  $\mu\text{M}$  nifedipine; on the stabilised new current plateau level, the addition of 10  $\mu\text{M}$  KBR on top of nifedipine did not cause further blockade. The washout of both compounds elicited a gradual current recovery. Figure 6b shows original  $I_{\text{Ca}}$  traces (note the initial fast inactivating  $\text{Na}^+$  current); the control  $I_{\text{Ca}}$  was reduced by around 20 % by nifedipine, and the KBR added on top of it did not cause further blockade. Conversely, when KBR was added first, nifedipine did not elicit further current blockade. Averaged pooled data on  $I_{\text{Ca}}$  blockade elicited by nifedipine or KBR, when added separately or in combination, are plotted in Fig. 6d, e. Note the occlusion by each compound of the current blockade elicited by the other.

In the experiment of Fig. 5c, we explored whether 1 and 10  $\mu\text{M}$  SEA affected  $I_{\text{Na}}$  or  $I_{\text{Ca}}$ . Neither in this example experiment nor in averaged experiments from different cells (Fig. 5d) did SEA affect  $I_{\text{Na}}$  or  $I_{\text{Ca}}$ .

### Effects of ouabain on the membrane potential

Through the inhibition of NKA, ouabain should favour the accumulation of  $\text{Na}^+$  ions in the cytosol, thereby altering the  $\text{Na}^+$  gradient across the plasmalemma and causing its depolarisation. Whether ouabain also elicited BCC depolarisation was explored with the patch-clamp technique in its current-clamp mode under the whole cell configuration, using a nominal  $0\text{Ca}^{2+}$  solution.





**Fig. 5** Effects of compounds KBR and SEA, on the whole cell sodium and calcium currents ( $I_{Na}$  and  $I_{Ca}$ ). BCCs were voltage-clamped at  $-80$  mV under the whole cell configuration of the patch-clamp technique. Inward currents were generated by 50-ms test depolarising pulses to 0 mV, given at 30-s intervals. The initial fast inactivating current is  $I_{Na}$  and the later noninactivating current is  $I_{Ca}$  (arrows in

panels **a** and **c**). KBR (panel **a**) or SEA (panel **c**) were perfused at the indicated concentrations for 2 min. In panels **a** and **c**, example current traces from two BCCs are displayed. Panels **b** and **d** are averaged plots of experiments as those shown in panels **a** and **c**. They are means  $\pm$  SEM of experiments (n) and cultures (N) shown in parenthesis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  with respect to control

In the example record of Fig. 7a, the initial resting membrane potential ( $V_m$ ) of the cell was around  $-50$  mV; cell perfusion with  $10 \mu\text{M}$  ouabain elicited a gradual depolarisation that reached around  $-35$  mV in 2 min. It is interesting that after ouabain washout, the depolarisation persisted or even increased, as ouabain is known to stably bind to the membrane  $\text{Na}^+/\text{K}^+$  ATPase [6]. In the cell of Fig. 7c, ouabain was added first, and then,  $10 \mu\text{M}$  nifedipine was added on top of ouabain, showing no effect on the ouabain-elicited depolarisation. In 12 BCCs from 2 different cultures, ouabain produced a gradual depolarisation that amounted to  $8 \pm 1$  mV after 3 min. In the presence of nifedipine and after washout, the depolarisation augmented by  $18 \pm 2$  mV and by  $28 \pm 2.8$  mV.

#### Elevation of the cytosolic calcium concentrations elicited by $2\text{Ca}^{2+}$ pulses in ouabain-treated BCCs

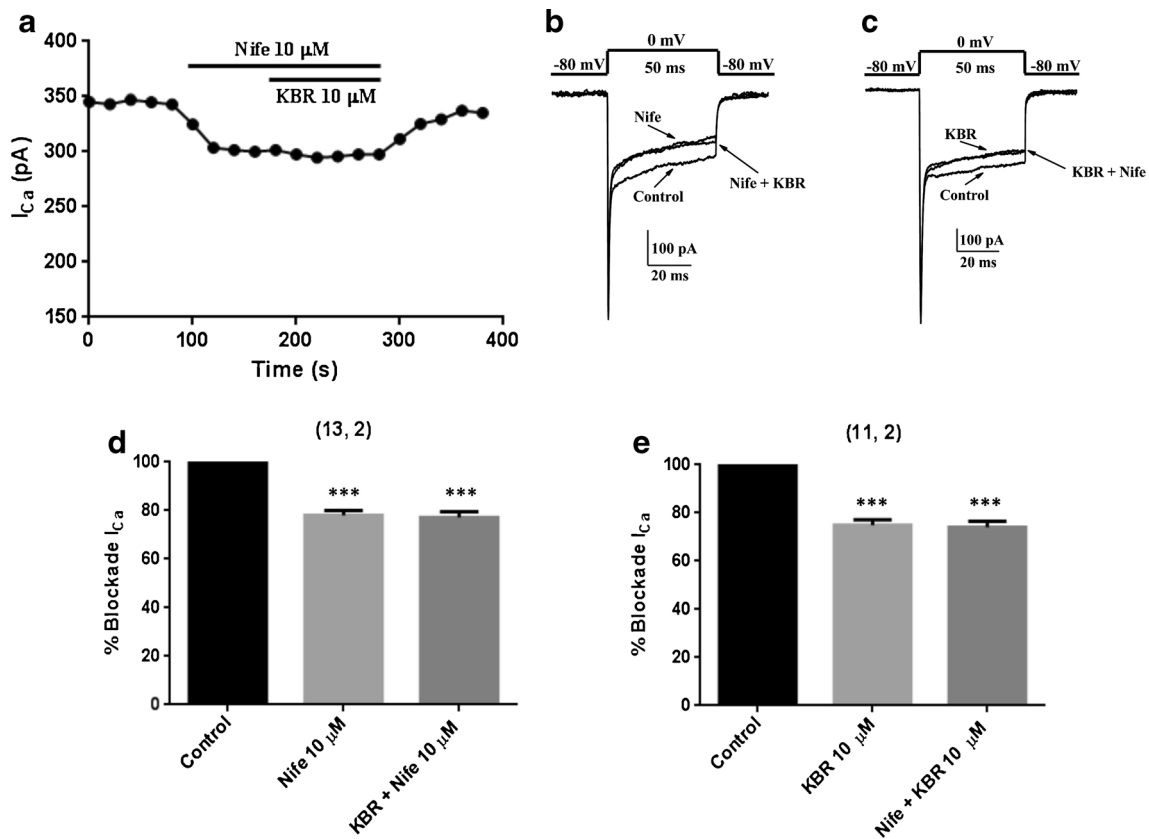
Changes in the  $[\text{Ca}^{2+}]_c$  transients elicited by  $2\text{Ca}^{2+}$  pulses, before and during exposure to ouabain of fluo-4-loaded BCCs, were studied following protocols similar to those used to monitor secretion (Figs. 1 to 4). This could orient on a possible correlation between  $[\text{Ca}^{2+}]_c$  and secretion changes.

In the example  $[\text{Ca}^{2+}]_c$  record of Fig. 8a, the cell that was being perfused with  $0\text{Ca}^{2+}$  was challenged with sequential 10-s pulses given with the  $2\text{Ca}^{2+}$  solution at 3-min intervals.

Ouabain at  $10 \mu\text{M}$ , perfused from pulse 3 onwards, augmented the  $[\text{Ca}^{2+}]_c$  from around 12 arbitrary fluorescence units (AFU) to 25 AFU at pulses 3 and 4. This augmentation continued until pulse 7 that reached around 27 AFU; from this pulse onwards, the  $[\text{Ca}^{2+}]_c$  was maintained at values of around 30 AFU. In a similar experiment (Fig. 8b), ouabain more than doubled the amplitude of the  $[\text{Ca}^{2+}]_c$  transient while  $10 \mu\text{M}$  nifedipine given on top of ouabain during pulses 8 to 10 drastically decreased the  $[\text{Ca}^{2+}]_c$  transient amplitude from 22 AFU to only 8 AFU. Upon nifedipine washout, the  $[\text{Ca}^{2+}]_c$  transient recovered its initial amplitude. Figure 8c shows a trace of  $[\text{Ca}^{2+}]_c$  transients produced by  $\text{Ca}^{2+}$  pulses in the presence of ouabain and the lack of effects of  $\omega$ -toxins (combined  $\omega$ -conotoxin GVIA at  $1 \mu\text{M}$  and  $\omega$ -agatoxin IVA at  $1 \mu\text{M}$ ). A graph summary of pooled normalised data from 24 cells is displayed in Fig. 8d. Note the drastic blockade exerted by nifedipine of these  $[\text{Ca}^{2+}]_c$  transients, the lack of effect of  $\omega$ -toxins and again, the blockade by nifedipine in the presence of  $\omega$ -toxins.

#### Discussion

The central finding of this study was the two-step time course of the catecholamine release responses triggered by repeated  $2\text{Ca}^{2+}$  pulses applied to BCCs perfused with a  $0\text{Ca}^{2+}$  solution



**Fig. 6** Occlusion experiments showing that the fraction of the whole cell inward  $Ca^{2+}$  current ( $I_{Ca}$ ) blocked by nifedipine (10  $\mu$ M) or KB-R7943 (KBR; 10  $\mu$ M) was the same. Patch-clamp experiments were made under conditions similar to those of Fig. 5. **a** Time course of  $I_{Ca}$  amplitude (ordinate in pA); compounds nifedipine (Nife) or KBR were perfused during the periods identified by the top horizontal lines. **b, c** Original current recordings showing the  $I_{Ca}$  traces (preceded by the fast

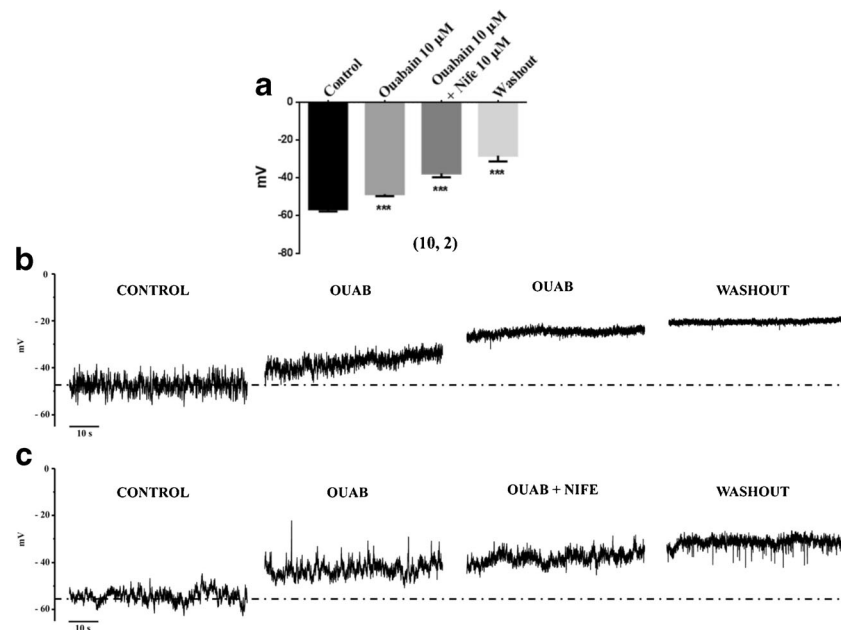
inactivating  $Na^{+}$  currents), and the blockade of nifedipine or KBR added alone and subsequently within the same cell, in combination. **d, e** Quantitative pooled data showing the blockade of  $I_{Ca}$  exerted by compounds Nife and KBR, added alone or in combination. These data are means  $\pm$  SEM of the number of cells and cultures shown in parentheses. \*\*\* $p < 0.001$  with respect to the initial control  $I_{Ca}$

containing ouabain: during the first 30 min, the initially tiny responses gradually increased to reach a stable steady-state plateau that lasted another 30 min. Because the BCCs exposed to ouabain underwent rapid depolarisation, we may reasonably conclude that the gradual delayed increase of secretory responses was not due to gradual cell depolarisation. This is corroborated by the observation that the secretory responses elicited by repeated  $35K^{+}/2Ca^{2+}$  pulses exhibited a gradual increase very much as that occurring with  $2Ca^{2+}$  pulses: the  $35K^{+}$ -elicited depolarisation [45] did not contribute to produce further depolarisation in cells already depolarised by ouabain.

Concordant with rapid depolarisation of ouabain-treated cells were the  $[Ca^{2+}]_c$  transients elicited by repeated  $2Ca^{2+}$  pulses that exhibited a plateau steady-state amplitude at few minutes after adding ouabain. Thus, we cannot attribute the gradual increase secretory responses to more  $Ca^{2+}$  entry with each  $2Ca^{2+}$  pulse. Therefore, the rate limiting step in causing a delay to reach a steady-state secretion responses is neither gradual time-dependent depolarisation nor gradual increases of  $[Ca^{2+}]_c$  transient amplitudes. It may likely be due to a time-

dependent increase of the rate of cytosolic vesicle transport from a large reserve pool of secretory vesicles (RP) toward a small subplasmalemmal ready-release vesicle pool (RRP) [42, 54]. In fact, using total internal reflection fluorescence microscopy (TIRFM), we found that ouabain favoured the accumulation of docked vesicles underneath the plasmalemma of BCCs [40].

Vesicle translocation from the RP to the RRP depends on mild elevations of the  $[Ca^{2+}]_c$ , which are subthreshold to trigger rapid exocytosis. This was first suggested at Neher's laboratory that used mild depolarisations to increase  $Ca^{2+}$  entry through VACCs, or histamine to release  $Ca^{2+}$  from the endoplasmic reticulum store (ER) of BCCs [58]. Other experimental strategies that were later on used to augment  $Ca^{2+}$ -dependent vesicle transport consisted in the stimulation with subthreshold nicotine [13], with acetylcholine pre-pulses [5] or the activation of the  $Ca^{2+}$ -induced  $Ca^{2+}$  release mechanism present in BCCs [4] by ouabain [40]. In our present experiments, the BCCs were intentionally perfused with nominally  $0Ca^{2+}$ , and thus, the contribution of the ER  $Ca^{2+}$  would be minimal. Thus,  $Ca^{2+}$  entry through VACCs during the



**Fig. 7** Ouabain causes a gradual and fast depolarisation of BCCs. The membrane potential ( $V_m$  in mV, ordinates) was recorded under the whole cell configuration of the patch-clamp technique, in the current-clamp mode. **b** An example record of the time-dependent depolarising effects of ouabain (OUAB, 10  $\mu$ M) that reached a maximum in about 3 min. In **c**,

another cell showing that the depolarising effect of OUAB is not affected by 10  $\mu$ M nifedipine (NIFE). In panel **a**, pooled data on the effects of ouabain, alone or with nifedipine, are shown. They are means  $\pm$  SEM of the number of cells and cultures shown in parentheses. \*\*\* $p < 0.001$ , with respect to the initial membrane potential

application of the  $2Ca^{2+}$  pulses could likely be responsible for both, the pre-exocytotic acceleration of vesicle transport during pulse intervals and the rapid exocytosis occurring during the application of  $2Ca^{2+}$  pulses.

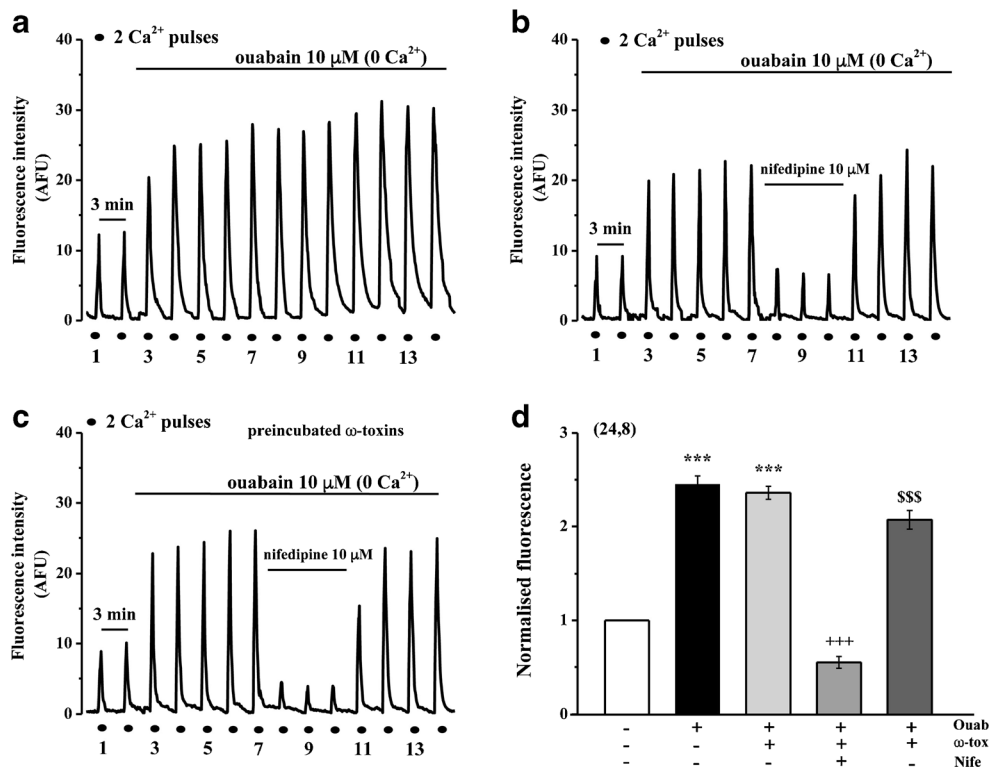
Now, the question concerns the membrane pathway used by  $Ca^{2+}$  to enter the cell during the  $2Ca^{2+}$  pulses. Chromaffin cells express various subtypes of VACCs [23, 37], including L, N and PQ channels. Near full blockade by nifedipine of the secretion triggered by  $2Ca^{2+}$  pulses excludes the non-L, N/PQ VACCs as contributors to this secretory response; the experiments performed with  $\omega$ -toxins that did not prevent the development of the ouabain-elicited gradual potentiation of secretion (Fig. 1d, e), strengthen the view that in ouabain-treated BCCs, the augmented secretion is attributable to a selective  $Ca^{2+}$  entry via L-type channels.

It is surprising that L-type channels remain open during the 60-min duration of an experiment using ouabain and the  $2Ca^{2+}$ -pulse protocol. This behaviour reminds that observed in a previous study also performed in BCCs that underwent sustained depolarisation with a  $0Ca^{2+}$  solution containing 100 mM  $K^+$ ; when challenged with 100 mM  $K^+$  containing 2 mM  $Ca^{2+}$ , these cells responded with a secretory response of around 150 nA that was near fully suppressed by nifedipine and drastically potentiated by FPL64176 and was unaffected by the combined treatment with  $\omega$ -conotoxin GVIA and  $\omega$ -agatoxin IVA [57]. Thus, it seems plausible that those earlier experiments with sustained  $K^+$  depolarisation of BCCs and the here reported experiments with ouabain have a similar

underlying mechanism, namely, the voltage-dependent inactivation of N/PQ channels, while L channels exhibit an extraordinary resistance to voltage-dependent inactivation. The experiments with repeated  $35K^+$  pulses in ouabain-treated cells, with secretion responses that are blocked by nifedipine but not by  $\omega$ -toxins, also support this conclusion (Fig. 3).

That ouabain elicits that cell depolarisation was predictable on the basis of its ability to inhibit the plasmalemmal NKA, thus favouring the gaining of  $Na^+$  by the cell [53]. The current-clamp experiment of Fig. 7b illustrates the property of ouabain to elicit a prompt membrane depolarisation. Although this behaviour does not exactly reproduce the sustained cell depolarisation chemically produced by high  $K^+$  [57], it likely explains the selective inactivation of N/PQ channels, and the isolation of the L channel-linked selective  $Ca^{2+}$  entry pathway in BCCs under prolonged exposure to ouabain. This is supported by the observation that enhanced  $[Ca^{2+}]_c$  elicited by  $2Ca^{2+}$  pulses in ouabain-treated BCCs was also truncated by nifedipine (Fig. 8b) and was unaffected by  $\omega$ -toxins.

A physiological explanation for the gradual accumulation of secretory vesicles at subplasmalemmal exocytotic sites is compatible with the hypothesis of a feedback link between the size of the RRP and the rate and capacity of the vesicle transport machinery through the cytosol, towards subplasmalemmal regions. Furthermore, the steady state of the secretion responses reached after about 30-min application of  $2Ca^{2+}$  pulses indicates that vesicle use and supply reached an equilibrium after 30 min of continued challenging of BCCs with the  $2Ca^{2+}$



**Fig. 8** Augmentation by ouabain of the elevations of the cytosolic  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_c$ ) elicited by  $2\text{Ca}^{2+}$  pulses. Cells were loaded with fluo-4 and subjected to experimental protocols similar to those used to monitor secretion. **a** This record on  $[\text{Ca}^{2+}]_c$  transients (expressed as  $\Delta$  arbitrary fluorescence units, AFU, in the ordinate) is from a BCC perfused with a nominal  $0\text{Ca}^{2+}$  solution and challenged with 10-s  $2\text{Ca}^{2+}$  pulses (Krebs-HEPES containing 2 mM  $\text{Ca}^{2+}$ ) applied at 3-min intervals (*bottom dots*). Ouabain was perfused during the time period indicated by the *top horizontal bar*. **b** This cell was perfused with

$0\text{Ca}^{2+}$  while ouabain and nifedipine were applied during the time period indicated by the *top horizontal bars*. **c** An original experiment similar to that of panel **b** but using  $\omega$ -toxins (1  $\mu\text{M}$   $\omega$ -conotoxin GVIA and 1  $\mu\text{M}$   $\omega$ -agatoxin IVA). **d** Averaged pooled data from experiments with protocol B and C. Data are means  $\pm$  SEM of the number of cells and cultures shown in parentheses; they are normalised as % of the  $\Delta$  AFU within each individual cell. \*\*\* $p < 0.01$  with respect to initial; +++ $p < 0.001$  with respect to ouabain; \$\$\$ $p < 0.01$  with respect to ouabain plus nifedipine

pulses. It may also suggest that vesicle use of the RRP during repeated cycles of exocytosis constitutes a positive feedback signal to gradually augment the rate of vesicle supply in the successive  $2\text{Ca}^{2+}$  pulses. This equilibrium, however, does not mean that the size of the RRP has reached its limit, because in the presence of L-type VACC activator FPL64176, the secretion elicited by  $2\text{Ca}^{2+}$  pulses near doubled.

Another point of physiological relevance is the potential contribution of the plasmalemmal NCX to the regulation of secretion in ouabain-treated BCCs. From a pharmacological optic, it was interesting that KBR fully blocked secretion (Fig. 4a); we attribute this effect to the blockade of L-channels that in BCCs, contribute about 20 % to the whole  $I_{\text{Ca}}$  [23]. The 20–30 % blockade of  $I_{\text{Ca}}$  elicited by KBR (Fig. 5b) may surely include the full blockade of L channels. In contrast, the more selective NCX blocker SEA-0400 that did not affect  $I_{\text{Ca}}$  (Fig. 5D), slightly decreased the secretory response elicited by  $2\text{Ca}^{2+}$  pulses under ouabain, and elicited a rebound secretory effect upon its washout. This is compatible with a regulatory role of the NCX that under ouabain depolarisation should be working under its reverse mode, thus

implying a greater  $\text{Ca}^{2+}$  entry through the reverse mode of the NCX and a greater secretion elicited by the  $2\text{Ca}^{2+}$  pulses [46]. From a pharmacological point of view, our data on  $I_{\text{Ca}}$  suggest that KBR clearly blocks the L-type channels of BCCs while SEA did not; thus, SEA seems to be more selective to inhibit the plasmalemmal NCX and should preferably be used, instead of KBR to block such  $\text{Ca}^{2+}$  transporter.

We conclude with a physiological note. To favour vesicle flow towards subplasmalemmal and exocytotic sites, to replenish the empty RRP with new vesicles, a mild  $[\text{Ca}^{2+}]_c$  elevation seems to be required. This was first experimentally tested either by applying to chromaffin cells mild depolarising pulses or through their challenging with histamine before evoking the exocytotic burst [58]. In this context, the experiments here reported are compatible with ouabain doing this task of causing mild  $[\text{Ca}^{2+}]_c$  elevations to augment vesicle flow and RRP refilling. Because the adrenal cortex is particularly rich in endogenous ouabain [34] and circulating ouabain is being considered as a hormone secreted by the adrenals as a pivotal factor in cardiovascular diseases [43], we are tempted to attribute physiological implications of the results here described. The



physiological role may imply a chronic exposure of adrenal medullary chromaffin cells to adrenal cortical ouabain; this exposure in chronic stress situations in which a higher secretion of cortical ouabain could cause a mild depolarisation of the adrenal medullary chromaffin cells to maintain vesicle flow and secretion by  $\text{Ca}^{2+}$  entry through noninactivating L-type VACCs. In fact, circulating ouabain in humans is increasing upon physical exercise, although at nanomolar concentrations [8]. However, in the natural microenvironment of chromaffin cells that are exposed to cortical hormones, ouabain secreted from the adrenal cortex may locally reach higher concentrations to favour intracellular vesicle flow and the secretion of adrenaline during prolonged stress.

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