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Ouabain enhances exocytosis through the regulation of calcium handling by the endoplasmic reticulum of chromaffin cells

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ABSTRACT

The augmentation of neurotransmitter and hormone release produced by ouabain inhibition of plasmalemmal Na^+/K^+-ATPase (NKA) is well established. However, the mechanism underlying this action is still controversial. Here we have shown that in bovine adrenal chromaffin cells ouabain diminished the mobility of chromaffin vesicles, an indication of greater number of docked vesicles at subplasmalemmal exocytotic sites. On the other hand, ouabain augmented the number of vesicles undergoing exocytosis in response to a K^+ pulse, rather than the quantal size of single vesicles. Furthermore, ouabain produced a tiny and slow Ca^{2+} release from the endoplasmic reticulum (ER) and gradually augmented the transient elevations of the cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) triggered by K^+ pulses. These effects were paralleled by gradual increments of the transient catecholamine release responses triggered by sequential K^+ pulses applied to chromaffin cell populations treated with ouabain. Both, the increases of K^+-elicited $[Ca^{2+}]_c$ and secretion in ouabain-treated cells were blocked by thapsigargin (THAPSI), 2-aminoethoxydiphenyl borate (2-APB) and caffeine. These results are compatible with the view that ouabain may enhance the ER Ca^{2+} load and facilitate the Ca^{2+}-induced-Ca^{2+} release (CICR) component of the $[Ca^{2+}]_c$ signal generated during K^+ depolarisation. This could explain the potentiating effects of ouabain on exocytosis.

1. Introduction

Thanks to its ability to inhibit Na^+ and K^+ transport across crab nerve membranes, the steroid digitalis drug ouabain played a fundamental role in the discovery of the sodium pump [1]. Since then, the only recognised receptor for ouabain (and other cardiac glycosides) is the plasma membrane NKA or sodium pump; this is directly responsible for the maintenance of the low intracellular Na^+/K^+ ratio by the active transport of these ions across the plasma membrane [2] using the hydrolysis of ATP to provide the necessary energy [3]. This serves to maintain a hyperpolarised membrane potential which is necessary for synaptic transmission, muscle contraction, cell excitability and many other functions requiring an asymmetric distribution of Na^+ and K^+ ions between the intracellular and extracellular compartments. The sodium pump can also drive secondary active co-/counter transporters, such as the plasmalemmal Na^+/Ca^{2+} exchanger (NCX); thus, by rising the cytosolic concentrations of Na^+ ($[Na^+]_c$) and $[Ca^{2+}]$ upon NKA inhibition, cardiotonic steroids produce their recognised cardiac inotropic and toxic effects [4] as well as their ability to augment neurotransmitter release [5,6].

We have known for many years that by itself, ouabain augments the rate of spontaneous catecholamine release from adrenal medullary chromaffin cells [7–10]. Furthermore, ouabain also enhances the secretory responses triggered by K^+ depolarising pulses applied to these cells [11,12]. This action has been explained on the basis of the classical "sodium pump lag hypothesis" proposed to explain the inotropic effects of cardiac glycosides [4,13]. Thus, inhibition by ouabain of NKA causes an increase of $[Na^+]_c$ which favours the activation of the NCX in its reverse mode, thereby augmenting the $[Ca^{2+}]_c$ that enhances secretion [11,14–16].

Two interesting findings attracted much interest in the field of cardiotonic steroids and NKA. One emerged at the laboratory of Mordecai Blaustein that found a cardiotonic steroid indistinguishable from ouabain in human plasma [17–19]. The other concerns the observation that at concentrations that are unlikely to inhibit the enzymatic function of NKA, cardiotonic steroids are capable of initiating several intracellular signalling pathways.

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These pathways mostly involve the release of Ca^{2+} from the ER via the inositol 1,4,5-trisphosphate receptor (IP_3R) [20]; in fact, there are data supporting the idea that NKA tethers the IP_3R into a Ca^{2+} regulatory complex [21,22]. Also, NKA was shown to co-localize with subplasmalemmal regions of the ER, forming the so-called plasmerosomes found in rat neurons, myocytes and astrocytes [23] as well as in chromaffin cells [24].

We present here a study focused to test the hypothesis that the well known effect of ouabain to enhance the release of catecholamine from chromaffin cells, is linked to the regulation by the cardiac glycoside of Ca^{2+} handling by the ER. With the combination of amperometric techniques to monitor online the exocytotic responses, with imaging techniques to study vesicle movement and the intracellular Ca^{2+} dynamics, we found that ouabain augmented the secretory responses elicited by K^+ depolarising pulses through a mechanism implying the mobilisation of Ca^{2+} from the ER.

2. Materials and methods

2.1. Isolation and culture of bovine chromaffin cells

Bovine chromaffin cells were isolated from adrenal glands of adult cows, following standard methods [25] with some modifications [26]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin and 50 μg/ml streptomycin.

2.2. On-line measurement of catecholamine release from perifused cells

For experiments to study catecholamine secretion, cells were plated on plastic Petri dishes (60 mm diameter) at a density of 10^6 cells/ml (5 million cells per dish). Cells were kept for 1–4 days at 37 °C in a water-saturated incubator, with a 5% CO_2/95% air atmosphere. Before the experiment, cells were gently scrapped off from the bottom of the dish with a rubber policeman, centrifuged and packaged in glass wool in the bottom of a 100 μl microchamber and perifused with Krebs-HEPES solution (composition in mM: NaCl 144; KCl 5.9; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 11; HEPES 10; pH 7.4) at the rate of 2 ml/min. The liquid flowing from the perifusion chamber reached an electrochemical detector model Metrohn AG CH-9100 Hersau, which monitors amperometrically the amount of catecholamines secreted. Cells were intermittently stimulated to secrete their catecholamines with 10-s pulses of a Krebs-HEPES solution containing 35 mM K^+ (with isosmotic reduction of Na^+). All solutions used for the experiments were kept at 37 °C using a thermostatic bath.

2.3. Amperometric recording of quantal catecholamine release at the single-cell level

Catecholamine release from single cells was measured by amperometry [27]. Electrodes were built as previously described [28]. The amperometer was connected to an interface (Powerlab/45P AD instruments, New Zealand) that digitized the signal at 10 kHz sending it to an Apple Macintosh Power PC computer that displayed it within the Chart v.4.2 software (AD instruments, New Zealand). The electrodes were calibrated following good amperometric practices [29]. The coverslips were mounted on a Nikon Diaphot inverted microscope, and were continuously perifused, by means of 5-way perifusion system with a common outlet, with a Tyrode solution composed by (in mM): 137 NaCl, 1 $MgCl_2$, 5.3 KCl, 2 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.3, adjusted with NaOH). The high K^+ solution (24 mM K^+) was prepared by replacing equiosmolar concentrations of NaCl with KCl. Experiments were performed at room temperature (24 ± 2 °C).

2.4. Measurement of vesicle mean square deviation of chromaffin granules using TIRFM

For experiments using Total Internal Reflection Fluorescence Microscopy (TIRFM), chromaffin cells in suspension were electroporated immediately after isolation, using a Nucleofector device according to the manufacturer protocol O-001 (Amaxa), using 1×10^6 cells and 2 μg cDNA expressing neuropeptide-y coupled to EGFP (NPY-EGFP), a gift from Dr. Wolfhard Almers [30]. Cells were plated at a density of 5×10^5 on 18 mm-diameter coverslips and placed in 6-well plates. Cells were used 24–48 h thereafter. After electroporation cells were explored with an inverted microscope (Zeiss 200M) through a 1.45 NA objective (alpha Fluor, 100X/1.45, Zeiss) and using an immersion fluid (n_{488} = 1.518; Zeiss). For evanescent field (EF) illumination, the expanded beam (488 nm) of an argon ion laser (Lasos, Lasertechnik GmbH, Germany) was band-pass filtered (488/10; Zeiss) and used to excite EGFP. The images were projected onto a back-illuminated CCD camera (AxioCam MRm, Zeiss) through a dichroic FT500 and band-pass filter (525/50 nm). Each cell was imaged using Axiovision (Zeiss) for up to 2 min with 0.8-s exposures at 1 Hz when illuminated under EF. Stacks of EF images were analysed and single NPY-EGFP-labelled vesicles were followed using Metamorph software (Molecular Devices, CA, USA). Structures larger than 0.5 μm or if they became oblong at any time were excluded from the analysis. We marked the position of each tagged vesicle and tracked their x–y position as a function of time. To calculate the maximum range of any given trajectory, we used the distance of the median (x, y) of the data set to the furthest point to define the radius of a circle that would encompass every data point. Similar to described by Steyer and Almers [31] and Gaidarov et al. [32] and referred from the total number of cells.

2.5. Measurement of $[Ca^{2+}]_{ER}$ changes in bovine chromaffin cells transfected with ER-targeted D1-cameleon

Transfection of bovine chromaffin cells was achieved using the Amaxa Nucleofector electroporation system according to manufacturer's instructions (A-033 protocol) with 2×10^6 cells in 100 μl of nucleofection solution containing 2 μg cDNA coding for cameleon-D1 (gift from Dr. Roger Tsien). Cells were plated in 12 mm-diameter coverslips at a density of 4×10^5 cells. After 3–4 days in culture, coverslips were set in the stage of an upright fluorescence microscope (BX51WI, Olympus) with an Olympus 40X objective. For ratiometric measurements, we used 440 nm excitation and 512 and 480 nm emission wavelengths. Filter wheel (440DF20), dichroic beamsplitters (455DRLP) and emission filters (480DF30 and 535DF25) were purchased from Omega Optical and Chroma Technologies (Brattleboro, VT, USA). Images were captured using a CCD camera (Sony, Japan). Synchronization of the filter wheel and CCD camera as well as raw data acquisition was performed with the Olympus Cell R software (version 2.6). Ratio of fluorescence 512/480 was calculated and normalised using MS Excel software (Microsoft Corporation, Redmod, Washington, USA). Experiments were performed at room temperature (24 ± 2 °C).

2.6. Measurement of $[Ca^{2+}]_c$ changes in bovine chromaffin cells with Fura-2

Cells were plated in 12 mm-diameter coverslips at a density of 50,000–100,000 cells per well. After 48 h, single-cell measurements of $[Ca^{2+}]_c$ were performed at room temperature in 2.5 μM fura-2 (Molecular Probes, Invitrogen, Barcelona, Spain) loaded cells