Full Paper

On the Mechanism of Levosimendan-Induced Dopamine Release in the Striatum of Freely Moving Rats

Gaia Rocchitta¹, M. Rosaria Delogu¹, Rossana Migheli¹, Luigi Solinas², Giuseppe Susini², Maria S. Desole¹, Egidio Miele¹, Maddalena Miele³, and Pier Andrea Serra^{1,*}

¹Department of Pharmacology and ²Institute of Anesthesiology, Faculty of Medicine, University of Sassari, viale S. Pietro 43B, 07100 Sassari, Italy

³Department of Psychiatry, St. Mary's Hospital, London, UK

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Abstract. The Ca^{2+} sensitizer levosimendan (LEV) improves myocardial contractility by enhancing the sensitivity of the contractile apparatus to Ca^{2+} . In addition, LEV promotes Ca^{2+} entry through L-type channels in human cardiac myocytes. In this study, which was performed using microdialysis, infusion of LEV at $0.25 \mu\text{M}$ for 160 min increased dopamine (DA) concentrations (up to fivefold baseline) in dialysates from the striatum of freely moving rats. Ca^{2+} omission from the perfusion fluid abolished baseline DA release and greatly decreased LEV-induced DA release. Reintroduction of Ca^{2+} in the perfusion fluid restored LEV-induced DA release. Chelation of intracellular Ca^{2+} by co-infusing 1,2-bis (*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM, 0.2 mM) did not affect basal DA release and scarcely affected LEV-induced increases in dialysate DA. In addition, co-infusion of the L-type ($\text{Ca}_v 1.1-1.3$) voltage-sensitive Ca^{2+} -channel inhibitor nifedipine failed to inhibit LEV-induced increases in dialysate DA, which, in contrast, was inhibited by co-infusion of the N-type ($\text{Ca}_v 2.2$) voltage-sensitive Ca^{2+} -channel inhibitor ω -conotoxin GVIA. We conclude that LEV promotes striatal extracellular Ca^{2+} entry through N-type Ca^{2+} channels with a consequent increase in DA release.

Keywords: levosimendan, microdialysis, striatal dopamine, calcium entry

Introduction

The Ca^{2+} sensitizer levosimendan (LEV) improves the function of stunned myocardium, cardiac performance in heart failure, and possibly the efficiency of myocardial work. In low concentration, LEV does not increase cytosolic free Ca^{2+} level, but improves myocardial contractility by enhancing the sensitivity of the contractile apparatus to Ca^{2+} (1–4). In human cardiac myocytes, however, LEV increased also L-type ($\text{Ca}_v 1.1-1.3$) Ca^{2+} current via phosphodiesterase-3 inhibition (5). Ca^{2+} signaling pathways in cells are complex and involve both extracellular Ca^{2+} entry and intracellular Ca^{2+} pool release (6). Depletion of intracellular Ca^{2+} stores is one of the major triggering mechanisms for the activation of Ca^{2+} entry during the generation of

receptor-mediated Ca^{2+} signals (7, 8).

The current view on the release of neurotransmitters in the CNS by Ca^{2+} -triggered synaptic vesicle exocytosis is that the process involves several steps and is controlled by a protein machinery that must prevent neurotransmitter release before Ca^{2+} entry into pre-synaptic terminals, and yet must rapidly induce release on Ca^{2+} influx (9). This sequence of events obviously underlies the spontaneous release of dopamine (DA) from dopaminergic terminals in CNS dopaminergic fields.

In light of the above findings, the study of the LEV effects on DA release in the striatum of freely moving rats was deemed of interest.

*Corresponding author. FAX: +39 79 228525
E-mail: pharmaco@ssmain.uniss.it

Materials and Methods

Drugs and suppliers

Levosimendan [Simdax™, 1 × 5 ml vial (2.5 mg/ml), lot 74-001-5W] was kindly supplied by Abbott (Milano, Italy); 1,2-bis (*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra (acetoxymethyl) ester (BAPTA-AM) was purchased from Calbiochem-Enalco (Milano, Italy), ω -conotoxin GVIA from Tocris Cookson (Bristol Avon, UK), and nifedipine from Sigma-Aldrich (Milano, Italy).

Animals

Male Wistar rats (Morini, R. Emilia, Italy), weighing between 280–330 g, were used in all experiments. The rats were maintained under standard animal care conditions (12:12 h light/dark cycle, lights coming on at 7 a.m.; room temperature of 21°C), with food and water ad libitum. Prior to the start of any experiment, the health of the rat was assessed according to published guideline (10). All procedures were specifically licensed under the European Community directive 86/609 included in Decreto No. 116/1992 of the Italian Ministry of Public Health.

Microdialysis probe construction

The striatal probe is a modification of the one previously described (11) and combines two independent microdialysis probes of concentric design with two separate inlets with two corresponding separate outlets (Fig. 1), which permits separate co-infusion of drugs and separate dialysate sample collection from the same intrastriatal site. Separate sample collection is useful when one or more drugs, which may have either pro-oxidant or antioxidant properties, are infused. In fact, separate sample collection may permit the distinction of neurochemical changes that occur in the extracellular compartment (“intra-striatum”) from those that may occur in the collected sample (“post-striatum”), as a result of a chemical reaction. The probes were constructed using two sections of plastic-coated silica tubing (diameter of 0.15 mm; Scientific Glass Engineering, Milton Keynes, UK), each placed in the center of semi-permeable polyacrylonitrile dialysis fibres (molecular cut-off weight of 12 kd, Filtral 16; Hospal Industrie, Meyzieu Cedex, France). Each probe had a final diameter of 0.22 mm. The tips of the dialysis fibres were sealed and joined using quick-drying epoxy glue. The two sections of silica tubing served as inlets; the outlets were also made with a section of plastic-coated silica tubing, positioned in the center of the polythene tubing. The semi-permeable membrane was coated with epoxy, leaving an active length of 4 mm. The diameter

Fig. 1. Drawing of a striatal probe combining two independent microdialysis probes of concentric design with two separate inlets and two corresponding outlets. Each probe has a final diameter of 0.22 mm. The semipermeable membrane has an active length of 4.0 mm. The diameter of the final probe is approximately 0.45 mm. The main drug is infused through the inlet 1a, conventionally indicated as the ipsilateral inlet, while another drug may be co-infused through the inlet 1b, conventionally indicated as the contralateral inlet. Both drugs diffuse through the 4 mm of the pertinent dialytic membrane and reach the extracellular compartment. Thereafter, the concentration of neurochemicals is determined separately in dialysates collected from both outlets (2a, conventionally indicated as the ipsilateral outlet, and 2b, conventionally indicated as the contralateral outlet).

of the final probe was approximately 0.45 mm.

Surgery

Stereotaxic surgery was performed under chloral hydrate (400 mg/kg intraperitoneally) anesthesia. The microdialysis probes were implanted in the right striatum using the following co-ordinates from the atlas of Paxinos and Watson (12): A/P +0.5 mm from bregma, –2.5 mm M/L, and –6.0 mm D/V from dura. Body temperature during anesthesia was maintained at 37°C by means of an isothermal-heating pad (Harvard Apparatus, Kent, UK). Following surgery, the animals were placed in large plastic bowls (50 × 55 cm), and maintained in a temperature- and light-controlled environment, with free access to food and water. Experiments were carried out 24 h after probe implantation with the animal in its home bowl. This arrangement allowed the rats free movement.

Microdialysis procedure

The composition of the Ringer solution used was as follows: 147.0 mM NaCl, 4.0 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂ (pH 6.0). A microinfusion

pump (CMA/100; Microdialysis, Stockholm, Sweden) pumped Ringer solution at a flow rate of $1.5 \mu\text{l}/\text{min}$ using two separate syringes connected to the inlets by a length of polythene tubing; every 20 min, $30\text{-}\mu\text{l}$ dialysate samples were collected manually in $250\text{-}\mu\text{l}$ micro-centrifuge tubes (Alpha Laboratories, Eastleigh, Hampshire, UK) attached to the outlets. Subsequently, a $20 \mu\text{l}$ aliquot of each collected dialysate was injected into each of two parallel analytical systems. Drugs were added to the Ringer solution and infused via the striatal probe implanted in the striatum.

Chromatographic analysis

DA and its acidic metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were quantified by HPLC with electrochemical detection as previously described (13), using an Alltech (Sedrano, Italy) 426 HPLC pump equipped with an injector (mod. 7725; Rheodyne, Rohnert Park, CA, USA); column ($15 \text{ cm} \times 4.6 \text{ mm}$, i.d.) (ODS80TM C18; Tosoh Haas, Stuttgart, Germany); electrochemical detector (mod. LC4B; BAS, Lafayette, IN, USA); and a PC-based analog-to-digital converter system on a Star Chromatographic Workstation (Varian, Walnut Creek, CA, USA). The mobile phase was 0.1 M citric acid, 0.1 M Na acetate, 0.1 mM EDTA, 9% MeOH, and 50 mg/l sodium octylsulphate ($\text{pH} = 2.9$); the flow rate was $1.3 \text{ ml}/\text{min}$. The first sample was collected after 60 min of stabilization (time 0), and then dialysates were collected at 20-min intervals for 40 min prior to the start of experiments.

Histology

Following the experiments, rats were killed with an overdose of chloral hydrate ($800 \text{ mg}/\text{kg}$ intraperitoneally). The location of each microdialysis probe was confirmed by post-mortem histology. Brains were fixed in formal saline and $50\text{-}\mu\text{m}$ coronal sections were made with a cryostat. The slices were stained with cresyl violet and examined under a microscope.

Statistical analyses

The concentrations in the dialysate were expressed in nM (DA) or μM (DOPAC and HVA) and given as the mean $\pm$ S.E.M. Drug effects on neurochemicals were statistically evaluated in terms of changes in absolute dialysate concentrations. Statistical significance was assessed using analysis of variance (ANOVA) for differences between groups and over time. Differences within or between groups were determined by paired or unpaired *t*-tests with Bonferroni multiple comparison adjustment.

Results

Effects of LEV on DA release and effects of Ca^{2+} omission

The concentration of LEV was chosen according to Follath et al. (14). Intrastriatal infusion of LEV ($0.25 \mu\text{M}$ for 160 min, $n = 4$), through an inlet conventionally indicated as ipsilateral inlet, induced a long-lasting increase in DA concentrations in dialysates collected from the contralateral outlet, with a peak (350% of baseline) after 80 min (Fig. 2). DOPAC and HVA (baseline values 1.24 ± 0.27 and $0.87 \pm 0.12 \mu\text{M}$, respectively) were not affected (data not shown).

Ca^{2+} -free Ringer solution was infused through both inlets from the beginning of the μM experiments. Omission of Ca^{2+} resulted in a decrease by about 95–100% of baseline DA concentrations. LEV ($0.25 \mu\text{M}$ for 160 min, $n = 3$) was thereafter infused through the ipsilateral inlet. Ca^{2+} omission greatly inhibited LEV-induced dialysate DA increases (Fig. 2). The LEV-induced dialysate DA increase was fully restored when LEV ($0.25 \mu\text{M}$ for 160 min, $n = 3$) was infused in Ca^{2+} -containing Ringer (Fig. 1).

Fig. 2. Effect of intrastriatal infusion of LEV on DA concentrations in dialysates from the striatum of freely moving rats and effects of Ca^{2+} omission or reintroduction on LEV-induced changes. LEV ($0.25 \mu\text{M}$, $n = 4$) was infused for 160 min (solid horizontal bar, a) through the ipsilateral inlet in normal Ringer solution. Following Ca^{2+} -free Ringer solution infusion through both inlets from the beginning of the experiments (open horizontal bar, b), LEV ($0.25 \mu\text{M}$) was infused for 160 min (solid horizontal bar, a) in either Ca^{2+} -free Ringer ($n = 3$) or Ca^{2+} -containing Ringer ($n = 3$) through the ipsilateral inlet. During LEV infusion, Ca^{2+} -free Ringer or Ca^{2+} -containing Ringer was infused through the contralateral inlet, respectively. Dialysates were collected from the contralateral outlet, at 20-min intervals, during drug infusion and for 80 min after discontinuation of drug infusion. Values are given as the mean $\pm$ S.E.M. * $P < 0.05$, compared with pertinent baseline values.

Effects of the endogenous Ca^{2+} chelator BAPTA-AM on LEV-induced DA release

To assess the role of endocellular Ca^{2+} stores, LEV effects were evaluated during co-infusion of the endogenous Ca^{2+} chelator BAPTA-AM (15).

Infusion of BAPTA-AM 0.2 mM for 160 min affected neither DA nor DOPAC and HVA dialysate concentrations (data not shown). BAPTA-AM (0.2 mM, $n = 4$) co-infusion was initiated through the contralateral inlet 40 min before LEV (0.25 μM , 160-min infusion) through the ipsilateral inlet and maintained until the end of experiments. BAPTA-AM co-infusion scarcely affected LEV-induced dialysate DA increases (Fig. 3).

Effects of the L-type (Ca_v 1.1–1.3) voltage-sensitive Ca^{2+} -channel inhibitor nifedipine or the N-type (Ca_v 2.2) voltage-sensitive Ca^{2+} -channel inhibitor ω -conotoxin GVIA on LEV-induced DA release

The extracellular Ca^{2+} -dependent effect of LEV on DA release prompted us to assess the type of channel involved in Ca^{2+} entry in dopaminergic terminals. For this purpose, either the L-type Ca^{2+} -channel inhibitor nifedipine or the N-type Ca^{2+} -channel inhibitor ω -conotoxin GVIA were co-infused with LEV. The concentrations of nifedipine and ω -conotoxin GVIA were chosen according to Serra et al. (11) and Bergquist et al. (16), respectively.

Fig. 3. Effect of intrastriatal BAPTA-AM co-infusion on LEV-induced increases in dialysate DA. LEV (0.25 μM , same group as in Fig. 2) was infused for 160 min (solid horizontal bar, a) through the ipsilateral inlet; BAPTA-AM (0.2 mM) was co-infused for 260 min (solid horizontal bar, b) through the contralateral inlet. BAPTA-AM co-infusion was initiated 40 min before LEV infusion. Dialysates were collected, at 20-min intervals, during drug infusion and for 80 min after discontinuation of LEV infusion. Values are given as the mean $\pm$ S.E.M. and refer to concentrations in dialysates from the contralateral outlet. * $P < 0.05$, compared with pertinent baseline values.

Nifedipine (10 μM , $n = 4$) co-infusion was initiated through the contralateral inlet 40 min before LEV (0.25 μM , 160-min infusion) through the ipsilateral inlet. Nifedipine co-infusion failed to affect LEV-induced dialysate DA increases (Fig. 4).

ω -Conotoxin GVIA (10 μM , $n = 4$) co-infusion was initiated through the contralateral inlet 20 min before LEV (0.25 μM , 160 min infusion) through the ipsilateral inlet. ω -Conotoxin GVIA co-infusion significantly inhibited LEV-induced dialysate DA increases (Fig. 4).

Discussion

LEV belongs to a new group of positive inotropic drugs, generically indicated as calcium sensitizer. Calcium sensitizers cause positive inotropic effects mainly through their stabilizing effects on the cardiac troponin C, which results in Ca^{2+} -sensitization (17). They may also exert positive inotropism by increasing L-type (Ca_v 1.1–1.3) Ca^{2+} current (18).

Several types of Ca^{2+} channels coexist to regulate neurotransmitter release in the CNS (16, 19). DA release from dopaminergic terminals has been shown to be largely dependent on the N-type (Ca_v 2.2) and P/Q-type (Ca_v 2.1) voltage-dependent Ca^{2+} channels, but not on L-type channels (19). In the present study, LEV-induced increases in dialysate DA was dependent on extra-

Fig. 4. Effect of intrastriatal ω -Conotoxin GVIA or nifedipine co-infusion on LEV-induced increases in dialysate DA. LEV (0.25 μM , same group as in Fig. 2) was infused for 160 min (solid horizontal bar, a) through the ipsilateral inlet; either ω -Conotoxin GVIA (10 μM , $n = 4$) or nifedipine (10 μM , $n = 4$) was co-infused for 180 min (solid horizontal bar, b) through the contralateral inlet. Dialysates were collected, at 20-min intervals, during drug infusion and for 80 min after discontinuation of LEV infusion. Values are given as the mean $\pm$ S.E.M. and refer to concentrations in dialysates from the contralateral outlet. * $P < 0.05$, compared with pertinent baseline values; + $P < 0.05$, compared with the LEV group.

cellular Ca^{2+} and independent of the intracellular Ca^{2+} store. In fact, Ca^{2+} omission from the perfusion fluid abolished baseline DA release and greatly decreased LEV-induced DA release, while reintroduction of Ca^{2+} in the perfusion fluid restored LEV-induced DA release. On the other hand, co-infusion of the endocellular Ca^{2+} chelator BAPTA-AM did not affect LEV effects. Most likely, LEV promotes Ca^{2+} entry through the N-type ($\text{Ca}_v 2.2$) voltage-sensitive Ca^{2+} channel, since co-infusion of the specific inhibitor ω -conotoxin GVIA greatly decreased LEV-induced DA release. Co-infusion of the L-type Ca^{2+} -channel inhibitor nifedipine did not affect LEV-induced DA release, as expected (19). These results demonstrate that LEV sensitizing effects are not restricted to cardiac L-type Ca^{2+} channels (5), but also affect striatal N-type Ca^{2+} channels. Research is in progress to evaluate whether LEV might affect R-type ($\text{Ca}_v 2.3$) and T-type ($\text{Ca}_v 3.1-3.3$) Ca^{2+} channels involved in the nigral somatodendritic DA release (20).

The mechanism underlying LEV-induced sensitization of striatal N-type Ca^{2+} channels is still unknown. It has been demonstrated (21–23) that exogenous nitric oxide (NO), generated from NO-donors, increases store-operated channel activity in hamster smooth muscle and lung fibroblast lines in vitro, with a mechanism related to S-nitrosylation of thiol groups of proteins, either on the channel itself or of proteins involved in its coupling to pool emptying. The effect of NO-donors was greatly potentiated by external Ca^{2+} removal, which activates store-operated channels. In a study submitted for publication, we showed that NO, generated from NO-donors, greatly promoted capacitative Ca^{2+} entry in striatal dopaminergic terminals in vivo, with a consequent exaggerated DA release, provided the depletion of internal Ca^{2+} stores, i.e., when store-operated channels were already activated. Presumptive activation of store-operated channels on dopaminergic terminals was achieved either by Ca^{2+} removal from the Ringer solution or by co-infusion of the endogenous Ca^{2+} chelator BAPTA-AM (unpublished observations). A mechanism related to an increase in Ca^{2+} entry through store-operated channels can be excluded for the LEV-induced increases in striatal DA release; in fact, the activation of store-operated channels, achieved either by Ca^{2+} removal from the Ringer or by co-infusion of the endogenous Ca^{2+} chelator BAPTA-AM, did not potentiate LEV effects.

In conclusion, LEV increases striatal DA release by promoting extracellular Ca^{2+} entry through N-type Ca^{2+} channels. The consequent increase in the central post-synaptic dopaminergic input might have clinical relevance in those neurodegenerative disorders in which a decrease in post-synaptic dopaminergic input occurs.

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