The adenylate cyclase activator forskolin potentiates the positive inotropic effect of the phosphodiesterase inhibitor milrinone but not of the calcium sensitizer levosimendan nor of its hemodynamically active metabolites: an apparent conundrum.

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ABSTRACT

OR-1855 and OR-1896 are two hemodynamically active metabolites of the inodilator levosimendan, with calcium sensitizing activity, but their mechanism of action is still not fully understood.

It has been previously reported that the positive inotropic effect of levosimendan is not potentiated by the adenylate cyclase activator forskolin whereas forskolin does potentiate the effects of the phosphodiesterase (PDE) inhibitor milrinone.

To ascertain whether the active metabolites follow the same pattern of levosimendan, the positive inotropic effects of OR- 1855 and OR-1896, were studied in guinea-pig isolated papillary muscle in the presence and absence of forskolin. OR-1855 and OR-1896 were also tested as inhibitors of PDE-III and PDE-IV.

Our result show that 0.1 μM forskolin did not potentiate the positive inotropic effect of either OR-1855 or OR-1896, as in the case of the parent compound levosimendan. As in previous studies, the positive inotropic effect of milrinone was markedly potentiated in the presence of forskolin.

From these data we propose an explanation for the divergent behaviour of the calcium sensitizing drugs and PDE inhibitors.

KEYWORDS: levosimendan, inotropy, phosphodiesterase inhibitor, mechanism of action, cAMP

1. INTRODUCTION

Levosimendan is an i.v. inodilator used in acutely decompensated heart failure,[1] in perioperative settings,[2] and in intensive care.[3] Levosimendan has a triple mechanism of action[4] with clinical effects including an increase in output and cardiac index and in an improvement of both systemic and pulmonary venous congestion.[5] The inotropic effect of levosimendan is driven by calcium sensitization of the contractile apparatus via a selective binding of levosimendan on the N-terminal of the cardiac isoform of troponin C.[6] Levosimendan, however, is also a potent phosphodiesterase (PDE) inhibitor with a uniquely high selectivity for PDE-III relative to PDE-IV.[7] There has been lengthy debate about whether the PDE inhibitory property of levosimendan plays a role in the inotropic effects and overall in the clinical effects of the drug and two lines of thought have been developed: (1) the presence of both PDE-III and PDE-IV in cardiomyocytes implies the existence of parallel cyclic adenosine monophosphate (cAMP) decyclization pathways. This redundancy would make a highly selective PDE-III inhibitor such as levosimendan unable to increase cAMP since the PDE-IV path would still be operational;[8] or (2) PDE inhibition by levosimendan does increase cAMP levels sufficiently to create a synergy with the calcium sensitizing effect but not enough to affect the contractile apparatus per se.[9]

In clinical settings, it has been shown that the hemodynamic effects of levosimendan are prolonged due to the formation of two hemodynamically active plasma metabolites, OR-1855 and OR-1896 (see their chemical structures in the supplementary material).[10] The pharmacokinetics and pharmacodynamics characteristics of those metabolites has been described in details[11] and their role in the clinical effects of levosimendan treatment has been discussed abundantly.[12]

OR-1896 exerts a positive inotropy effect in *ex vivo* models[13] and inhibits PDE-III selectively in purified enzyme preparations.[14] In our present research, we sought to shed

61 further light on the mechanism of action of both metabolites and understand which effect(s)
62 underpin their inotropic properties.
63 In a previous report[15] the positive inotropic effect of the parent compound
64 levosimendan, seen as increase of contraction force, was not potentiated by forskolin, a
65 labdane diterpene derived from geranylgeranyl pyrophosphate commonly used to increase the
66 levels of cAMP by stimulation of adenylate cyclase,[16,17] whereas forskolin did potentiate the
67 effects of the classic PDE inhibitor, milrinone, which has inhibitory effects on both PDE-III
68 and PDE-IV.[18]
69 The aim of this study was to investigate whether the positive inotropic effects of
70 OR-1855 and OR-1896 follow the behaviour of the parent compound levosimendan and are
71 not potentiated by forskolin, or behave as the PDE inhibitor, milrinone.
72
73 2. MATERIALS AND METHODS
74 2.1 Chemicals
75 The compound used were levosimendan, batch LS, Orion Pharma; OR-1855, batch LS, Orion
76 Pharma; OR-1896, batch L7, Orion Pharma; Milrinone, batch LS, Orion Pharma; Forskolin,
77 Lot B25975, Calbiochem-Novabiochem Corp, La Jolla, CA, USA. All the test compounds
78 were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were diluted so that the final
79 DMSO concentration was 0.4% throughout the experiment.
80
81 2.2. Phosphodiesterase inhibition
82 Highly purified PDE-III and PDE-IV isozymes were isolated from human platelets and a
83 promonocytic cell line of patients with myeloid leukaemia (U-937), respectively, according to
84 published methods.[19] In brief, the supernatant fraction of the tissue homogenate was added to
85 a diethylaminoethanol-sepharose column and then eluted with a linear sodium acetate gradient

buffer. Collected fractions with peak PDE activities were analyzed for cAMP PDE activity. Purified PDE isozymes were incubated at 30°C for 30 min in a reaction mixture containing [3H]-cAMP (0.1 μM) and cAMP (0.1 μM) in the presence or absence of the test compounds. The amount of [3H]-5'-AMP regarded as a degradation product, was determined by using liquid scintillation detection as described previously.[20] Inhibitory assays were performed in duplicates.

2.3. Animals

The present study was performed in accordance with the guidelines of the Council of Europe and the US National Research Council. Approval was granted by the Animal Ethics Committees of Orion Pharma, Finland. Adult guinea-pigs of either sex (Dunkin Hartley, purchased from Mollegaard Breeding Center LTD., Denmark, or Crl:(Charles River, Germany), weighing 300- 400 g were used. Guinea-pigs were housed at 20 ± 1 °C with relative humidity of 50 ± 10%. Light-dark cycle was adjusted with lights on from 06.00 to 20.00h. The guinea-pigs were kept on a standard guinea-pig diet (Altromin 3120) and tap water ad libitum.

2.4. Papillary muscle preparations

Guinea-pigs were killed by a blow on the skull and the heart was excised. Right ventricular papillary muscle was dissected and rinsed in ice-cold Tyrode solution. Thereafter the papillary muscle was mounted for the measurement of isometric force in organ baths containing modified Tyrode solution (37°C) bubbled with carbogen (95% 0_2, 5% CO_2). The composition of the Tyrode solution was 135 mM NaCl, 1 mM $MgCl_2 \times 6H_2O$, 5 mM KCl, 2 mM $CaCl_2 \times 2H_2O$, 15 mM $NaHCO_3$, 1 mM $Na_2HPO_4 \times 2H_2O$, 10 mM glucose, at pH 7.35±0.05. The volume of the open horizontal chamber was 1 ml and the flow rate of the bathing solution

flowing through the chamber was 5 ml/min. The papillary muscle (<1 mm in diameter) was stretched horizontally between a force-displacement transducer (FT 0.3 C) and a needle fixed to the bottom of the chamber. The papillary muscle was electrically stimulated (Stimulator model S 48 F, Grass Instruments) at 1 Hz with rectangular pulses. The pulse duration was 4 ms. The stimulation strength was twice the threshold voltage.

2.5. Experimental procedure

After a stabilisation period of 60 min, 0.1 μM forskolin was added to the bathing solution (no addition in control experiments). After a further period of 30 min, a test compound (levosimendan, OR-1855, OR-1896, or milrinone) was added to the bathing solution at a starting concentration of 0.03 μM. (see an example of trace in **Figure 1**). Thereafter, the concentration of the test compounds was increased to 0.1, 0.3, 1, 3, 10, and 30 μM at 10 min intervals. The highest two concentrations were not tested for levosimendan, for solubility reasons (see the dosing schedules in **Figure 2**). All the experiments were carried out at 37 °C. The baselines values were measured at time "0" (as in Figure 2), i.e., immediately before the first addition of the test compounds. The baseline in experiments with forskolin were thus measured 30 minutes after the addition of 0.1 μM forskolin, and immediately before the first addition of the test compounds. We selected the concentration of forskolin based on a previous study on the positive inotropic action of the drug by Metzger H and Lindner E.,[21] assuming that the induced cAMP activation would be maintained from the beginning to the end of the papillary muscle contraction experiments as described previously.[22] The increase of contraction force from the baseline during the up-titration of every test compound was measured and analysed.

2.6. Statistics

Results obtained from five to nine experiments were combined and expressed as mean ± SEM. Differences between and within test groups were analysed by using Two Way Repeated Measures ANOVA followed by the Šídák test (Prism 9.1.0, GraphPad, CA, USA). A p-value <0.05 was considered statistically significant.

3. RESULTS

The baseline contraction force values of the guinea-pig papillary muscle preparates are shown in **Table 1**. No significative differences are seen between the experiments with forskolin and without forskolin.

OR-1855 and OR-1896 increased the inotropy of guinea-pig papillary muscle from baseline by maximum values of 312 ± 118 mg (n=5) and 341 ± 82 mg (n=8), respectively (**Figure 3**). The presence of forskolin 0.1 μM, did not potentiate significantly the positive inotropic effect of either compound: the maximum increases in contraction force in presence of the adenylate cyclase stimulant were 265 ± 62 mg (n=5) for OR-1855 and 334 ± 31 mg (n=6) for OR-1896.

For levosimendan, the maximum increases in contraction were 331 ± 58 mg (n=5) and 393 ± 69 mg (n=6) in the absence and presence of forskolin, respectively (n.s.). The maximal force increase from baseline with milrinone in the absence of forskolin was 219 ± 42 mg (n=5). That effect was significantly potentiated by forskolin (393 ± 69 mg; n=6) ($p<0.005$).

The IC_{50} for PDE-III and PDE-IV were calculated from the relevant dose-dependent inhibition curves of the four compounds (see **Table 2**). The PDE-III to PDE-IV IC_{50} ratio was also calculated; those values, reflecting selectivity of inhibition of PDE-III are 7619 for levosimendan, 3043 for OR-1986, 100 for OR-1855, and 39 for milrinone.

4. DISCUSSION

The adenylate cyclase activator forskolin increases intracellular cAMP level and thereby stimulates cAMP-dependent protein kinase A, which in turn increases calcium current[23] and enhances contraction force. On the other hand, the positive inotropic effect of some PDE inhibitor is potentiated by forskolin as previously demonstrated for instance with milrinone.[15] The two major plasma metabolites of levosimendan, OR-1855 and OR-1896 are thought to exert a positive inotropic activity by calcium sensitization of troponin C in the cardiomyocyte contractile apparatus. Nevertheless, these metabolites also inhibit the PDE-III isozyme in a highly selective manner in purified enzyme preparations.

One can hypothesize that the combination of the three aforementioned mechanisms (activation of adenylate cyclase, inhibition of phosphodiesterase, and calcium sensitization) would lead to an increased positive inotropy. Moreover, if these pathways are independent than their effects will be strictly additive. If there were to be any overlap (and hence non-additive effect on inotropy) between these mechanisms it might most likely arise between the two (activation of adenylate cyclase and inhibition of phosphodiesterase) sharing a common factor, *i.e.,* cAMP.

Our findings that the positive inotropic effects of OR-1855 and OR-1896 were not additive to the effects of forskolin (as was also the similarly as in the case or of their parent compound levosimendan) while whereas the effect of milrinone was, would appear to diverge from the above hypothesis.

One possible reason for the lack of contribution of adenylate cyclase stimulus to the inotropic effects of levosimendan and its metabolites is their high selectivity in PDE-III inhibition over the PDE-IV isoenzyme. It has been suggested that both PDE-III and PDE-IV should be inhibited to high levels in order to increase the amplitude of the intracellular calcium transient,[24] because an uninhibited PDE isozyme (i.e., PDE-IV in this case) can

potentially offset any effect from the inhibition of the other isoform (i.e., PDE-III). In keeping with this proposition, milrinone, which inhibits both isoenzymes, was potentiated by forskolin in our experiments. Accordingly, the effect of milrinone on intracellular cAMP and calcium concentrations is more prevalent than that for levosimendan.[15]

It is to also to note that in previous studies, that levosimendan induced NO production but that co-stimulation with cilostazol (another PDE-III inhibitor) failed to potentiate the effects of levosimendan on NO release in coronary endothelial cells.[25] This also speaks to a selective inhibition of PDE-III by levosimendan.

5. CONCLUSION

Like their parent compound levosimendan, the metabolites OR-1855 and OR-1896 have a positive inotropy effect which is not potentiated by forskolin. Conversely, the inotropic effect of the PDE-III/PDE-IV inhibitor milrinone is potentiated by adenylate cyclase activation. This different behaviour could be explained by the fact that positive inotropic effects evoked by milrinone or by levosimendan and its active metabolites are exerted via different mechanisms of action with different roles for cAMP.

The oral formulation of levosimendan is currently under scrutiny as treatment of pulmonary hypertension associated with heart failure[26] and the role of the active metabolites is paramount in this new pharmacokinetic/pharmacodynamics situation. Hence, the full characterization of OR-1855 and OR-1896 mode of action and pharmacology is of the outmost importance.

6. LIMITATIONS

In the study we used papillary muscles with diameter ≤1 mm. Diameters of individual preparates varied, however, as did the contraction force of induvial samples. This is why we

used the increase of force from baseline for our analysis. These preparations have intrinsic problems, such as a radius-dependent performance and the possibility that the core of the muscle bundle is hypoxic or even anoxic, However, radius-dependent decline of performance in isolated cardiac muscle does not reflect inadequacy of diffusive oxygen supply.[27]

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CONTRIBUTION

JL and PP designed the research study. JL performed the research. PP contributed essential reagents or tools. JL, PP, ZP and EG analyzed the data. PP and ZP wrote the paper.

DISCLOSURES

PP and JL are full time employee of Orion Pharma where the inotrope levosimendan was discovered and developed. Both EG and ZP have received, in the latest 5 years, either research grants and/or speaker honoraria and/or support for conference attendance from Orion Pharma.

Table 1: Baseline contraction force values of guinea-pig papillary muscle preparate

Study compound	**without Forskolin**			**with Forskolin**			
	Mean, mg	SEM	n	Mean, mg	SEM	n	difference
Levosimendan	260	47	5	238	32	6	ns
OR-1896	315	51	8	297	34	6	ns
OR-1855	327	38	5	354	58	5	ns
Milrinone	200	12	5	299	30	6	ns

233 **Table 2**: PDE-III/PDE -IV IC_{50} for OR-1896, OR1855, and levosimendan and for milrinone

Compound	**PDE-III, µM**	**PDE-IV, µM**	**PDE-IV vs PDE-III IC_{50} ratio**
Levosimendan	0.0021	16	7619
OR-1896	0.094	286	3043
OR-1855	5	500	100
Milrinone	0.45	17.5	39

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LEGENDS TO THE FIGURES

Figure 1

Example of tracing of papillary muscle contraction. The papillary muscle was electrically stimulated at 1 Hz with rectangular pulses of 4 ms. The stimulation strength was twice the threshold voltage. After a stabilisation period of 60 min, 0.1 µM forskolin was added to the bathing solution After a further period of 30 min, milrinone was added to the bathing solution at increasing concentrations.

Figure 2

Dosing schedule. The same color coding is used also in Figure 3.

Figure 3

Positive inotropic effect of levosimendan (upper left panel, yellow hexagons), OR-1896 (upper right panel, green dots), OR-1855 (lower left panel, blue triangles) and milrinone (lower right panel, red squares) in the presence and the absence of forskolin (0.1 µM) in guinea-pig isolated papillary muscle. Shown are mean changes of twitch tension ± SEM from the baseline levels. An asterisk (*) indicates a statistical significant difference ($p<0.05$) from the baseline level. A dagger (†) indicates a statistical significant difference ($p<0.05$) between the groups with and without forskolin. Data were analysed for statistical differences using two-way ANOVA followed by the Šídák test (Prism 9.1.0, GraphPad, CA, USA).

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372 **FIGURE 1**

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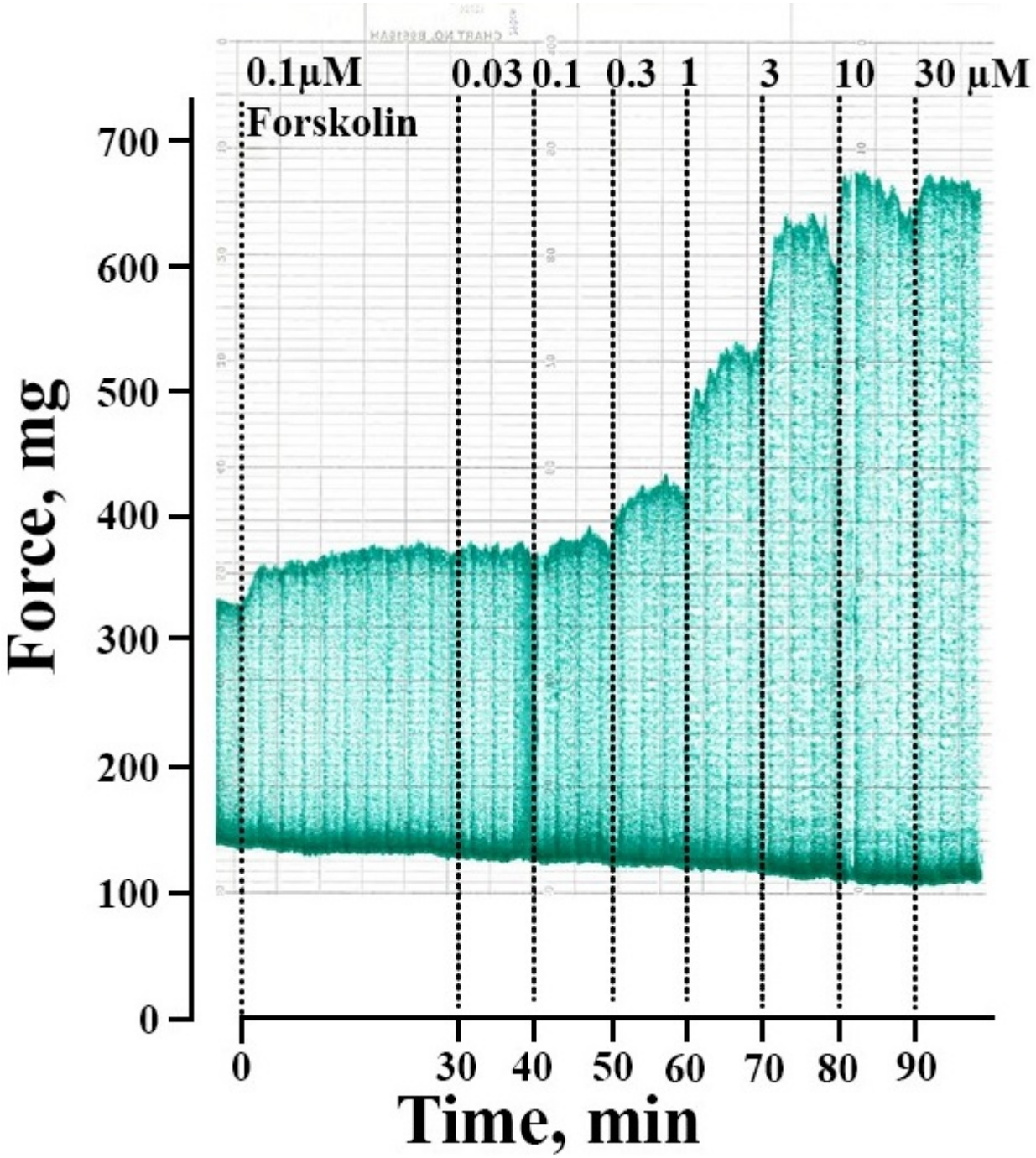

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376 **FIGURE 2**

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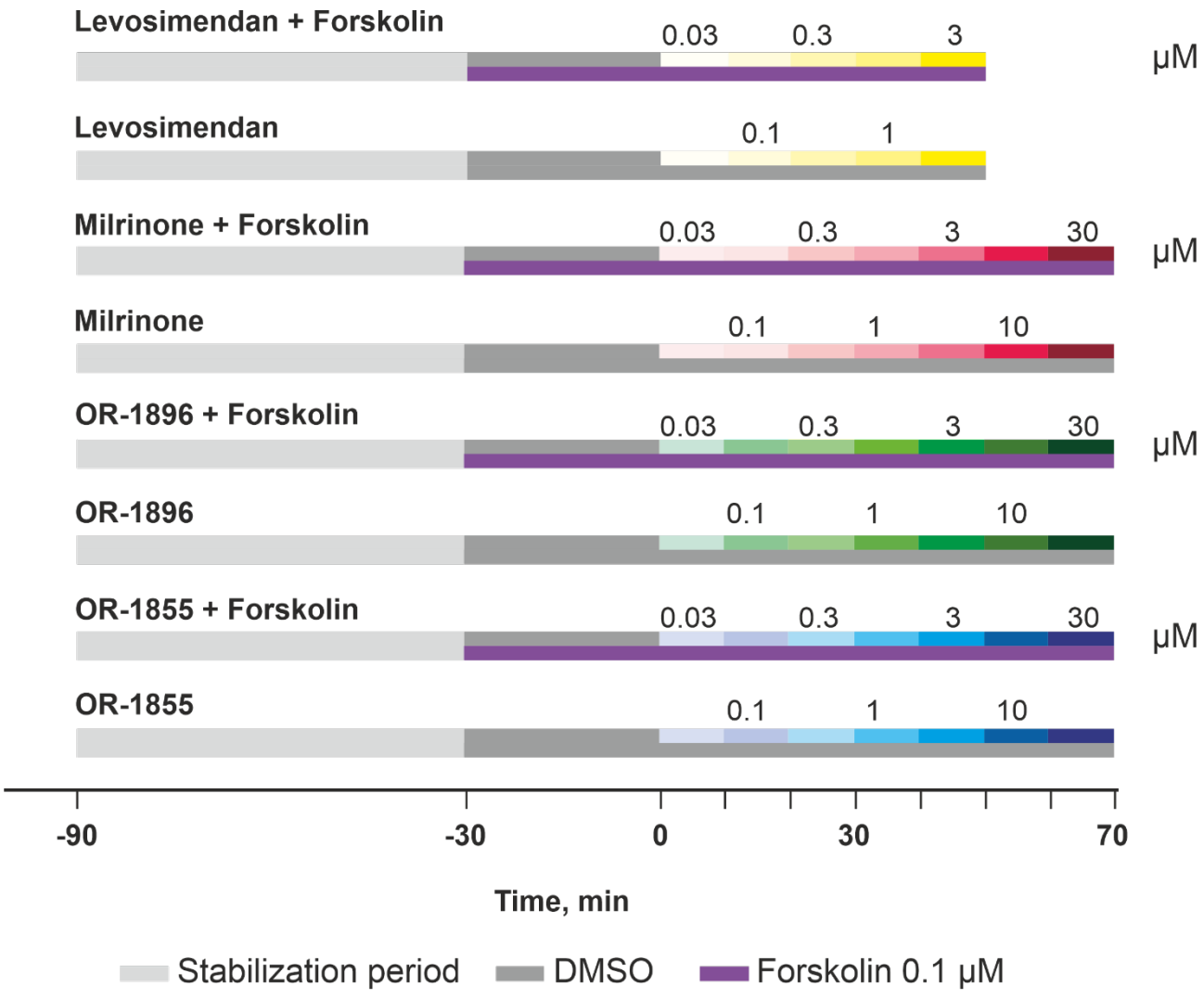

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379 **FIGURE 3**

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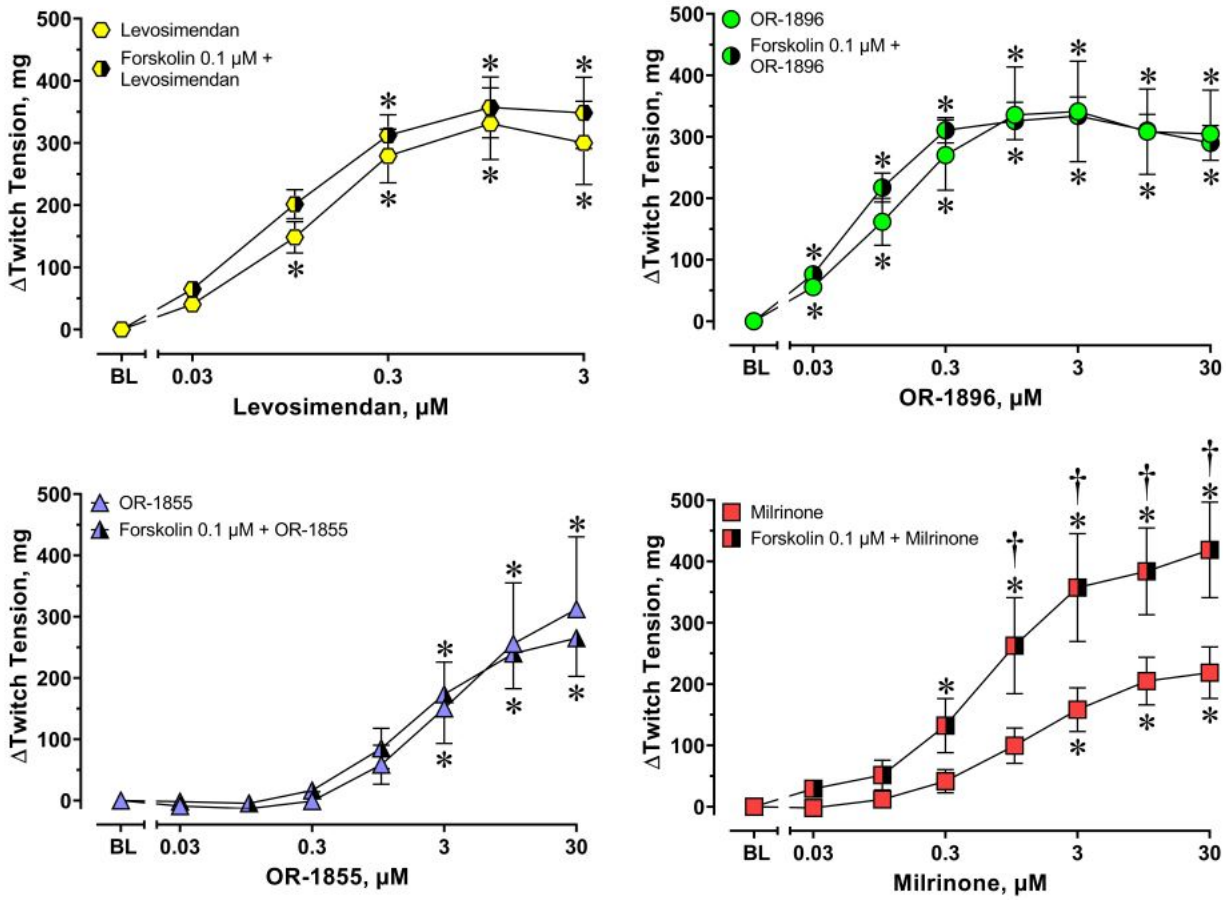

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383 **SUPPLEMENTARY MATERIAL**

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385 **CHEMICAL STRUCTURES**

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