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Pharmacological Effects of ATI22-107, a Novel Dual-Pharmacophore, on Myocyte Calcium Cycling and Contractility

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ABBREVIATIONS: ATI22-107, 2-(2-{2-[2-Chloro-4-(6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl)-phenoxy]-acetylamino}-ethoxymethyl)-4-(2-chloro-phenyl)-6-methyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid dimethyl ester); FFR, force frequency relation;

phosphodiesterase 3, PDE-III; ACE, angiotensin converting enzyme; NEP, neutral endopeptidase; SR, sarcoplasmic reticulum; LTCC, L-type Calcium Channel

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Abstract

Historically, inhibitors of type III phosphodiesterases (PDE-III) are effective inotropes in mammalian myocardium, but their clinical utility has been limited by adverse events, including arrhythmias that are considered to be due to Ca^{2+} overload. ATI22-107, a novel, dual-pharmacophore compound, was designed to simultaneously inhibit the cardiac phosphodiesterase (PDE-III) and produce inotropic effects, while inhibiting the L-Type Calcium channel (LTCC) to minimize increases in diastolic Ca^{2+}. We compared the effects of ATI22-107 and Enoximone, a pure PDE-III inhibitor, on the Fluo-3 calcium transient in normal feline ventricular myocytes and trabeculae. Enoximone-induced dose-dependent increases in peak $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, T50, and T75. ATI22-107 demonstrated similar dose-dependent increases in peak $[Ca^{2+}]_i$ at 300 nM and 1.0 μM doses with no further increases at higher doses. Throughout the dosing range, ATI22-107 induced much smaller, if any, increases in diastolic $[Ca^{2+}]_i$, T25, and T75. Current measurement of LTCC via patch clamp techniques revealed dose dependent decreases in LTCC current with increasing dose of ATI22-107 thereby validating the dual functionality of the drug that has been proposed in this study. Studies in isolated trabeculae demonstrated that enoximone-induced a dose-dependent augmentation of the entire force-frequency relation (FFR) in normal myocardium, whereas augmentation of contractility was only observed at lower stimulation frequencies with ATI22-107. These results demonstrate the effects of the LTCC antagonizing moiety of ATI22-107, and suggest that the novel simultaneous combination of PDE-III and LTCC inhibition by one molecule may produce a favorable profile of limited inotropy without detrimental effects of increased diastolic $[Ca^{2+}]_i$.

Introduction

In the past decade, novel and beneficial pharmacological effects from synthesized dual-pharmacophore compounds have spawned a legitimate interest in this therapeutic approach in various areas of medicine. Studies support the concept that dual-pharmacophores with two distinct pharmacologically-active moieties can be synthesized to exploit their respective benefits in a collaborative manner**.** Among these studies, the majority have been investigations for the pharmacologic intervention of inflammation, Alzheimers disease, hypertension, myocardial hypertrophy, and cancer (Farina NK, 2000; Chodjania et al., 2002; Yamamoto et al., 2002; Auvin et al., 2003; Toda et al., 2003; Uddin et al., 2003). An example of one such advance is the successful combination of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) inhibition moieties into a single agent. It has been shown that ACE/NEP inhibitor dual-pharmacophore compounds offer significant developmental and clinical advantages over combination therapy, thereby absolving the need to titrate the dose of two different agents simultaneously, avoiding the necessity to match the pharmokinetics of two independent therapies, and a simpler toxicology profile. Moreover, reports have shown that dual ACE/NEP inhibitors are providing compounds with augmented potency, duration of action, and improved oral bioavailability(Norton et al., 1999; Farina NK, 2000; Chodjania et al., 2002). To our knowledge, there have been no investigations of potential dual-pharmacophore technology applications to directly improving myocardial performance in heart failure.

Despite improvements in cardiac contractility, inotropes utilized to date have not been successful in decreasing mortality, due in large part to increases in sudden death(Teerlink et al., 2000). In fact, recent studies suggest that excessive sarcoplasmic reticulum (SR)

Ca^{2+} load may contribute to ventricular arrhythmias in failing hearts (Sipido et al., 2000) and could provide an explanation for increases in sudden death caused by existing inotropes, especially PDE-III inhibitors (Naccarelli GV, 1989). Nevertheless, as recently reviewed, depressed cardiac contractility and reduced contractility reserve represent fundamental features of the pathophysiology of progressive heart failure (Houser and Margulies, 2003). Therefore, the development of novel new inotropes is still relevant.

Using proprietary techniques, a novel dual-pharmacophore compound, (2-(2-{2-[2-Chloro-4-(6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl)-phenoxy]-acetylamino}-ethoxymethyl)-4-(2-chloro-phenyl)-6-methyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid dimethyl ester), also called ATI22-107, has been developed with distinct chemical moieties that inhibit phospodiesterase III (PDE-III) and the L-type Calcium Channel (LTCC). We hypothesized that ATI22-107 would preserve the inotropic effects of a pure PDE-III inhibitor while minimizing the increased diastolic calcium levels by antagonizing the LTCC. To address this hypothesis, we compared in vitro pharmacologic actions of a pure PDE-III inhibitor, (1,3-dihydro-4-methyl-5-[4-(methylthio)-benzoyl]-2H-imidazole-2-one), also called Enoximone with ATI22-107 in isolated cardiac myocytes and thin trabeculae from normal feline hearts. Our findings demonstrate clear distinctions between the two compounds and indicate that ATI22-107 does indeed exhibit moderate inotropic activity while simultaneously enhancing rates of myocardial $[Ca^{2+}]_i$ decay and preventing increases in diastolic calcium $[Ca^{2+}]_i$.

Methods

Pharmacology of ATI22-7 and PDEIII/LTCC specificity. The titled compound ATI22-107(2-(2-{2-[2-Chloro-4-(6-oxo-1,4,5,6-tetrahydro-pyridazin-3-yl)-phenoxy]-acetylamino}-ethoxymethyl)-4-(2-chloro-phenyl)-6-methyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid dimethyl ester) possesses an inhibitory activity against PDEIII and LTCC and is a patented dual pharmacophore compound with a published molecular structure (Hamilton and Leighton, 2003). In order to ensure the PDEIII and LTCC inhibitory effects of ATI22-107 we performed separate PDEIII and LTCC inhibition assays as described previously (Lee et al., 1984; Weishaar et al., 1986).

Animal preparation and Myocyte Cell Isolation. Normal adult felines were used for these studies. Animals were handled in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, revised 1996). Animals were anesthetized with pentobarbital and feline left ventricular myocytes were isolated as previously described.(duBell and Houser, 1989)

Intracellular Calcium ($[Ca^{2+}]_i$) in Isolated Myocytes. Freshly isolated myocytes were loaded with FLUO-3 AM (Molecular Probes, Eugene, Oregon), at 4-10 μM final concentration, in the presence of 1mM Ca^{2+}. Myocytes were placed in a chamber on the stage of an inverted microscope and was superfused at 1-2 ml/min with Tyrode's Solution (150mM NaCl, 5.4mM KCl, 1mM CaCl, 1.2mM MgCl, 10mM Glucose, 2mM pyruvate, 5mM HEPES, pH 7.4, 37ºC). Calcium- tolerant, rod-shaped myocytes were chosen based

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on their appearance, including the absence of membrane irregularities and spontaneous contractions. FLUO-3 was excited at 480 nm with xenon light and the emitted light was split with a 505 nm dichroic mirror. The emission at 530 nm was recorded to represent the cytosolic $[Ca^{2+}]_i$ transient. Myocytes were field stimulated at 1.0 Hz and were exposed to increasing doses (0μM, 0.3 μM , 1.0 μM, 3.0μM, and 10 μM) of either enoximone or ATI22-107. For every cell, 1.5 to 2 minutes of equilibration time was allowed upon each increase in dose until a steady state of the peak $[Ca^{2+}]_i$ was achieved. The $[Ca^{2+}]_i$ signal was recored and saved on a computer for later analysis using pClamp software (Axon Instruments, CA). Variables derived from raw $[Ca^{2+}]_i$ transients included peak $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, time to 50 percent decay of the $[Ca^{2+}]_i$ transient (T_{50}) and time to 75 percent decay of the $[Ca^{2+}]_i$ transient (T_{75}).

Measurement of LTCC Currents ($I_{Ca,L}$). Myocytes were studied in a chamber mounted on an inverted microscope (Nikon, Japan) and were initially perfused with a normal physiological salt solution containing (in mmol/L): NaCl 150, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1.2, glucose 10, sodium pyruvate 2, and HEPES 5 (pH7.4) at 37^{o}C. Low resistance (1-4 MΩ) patch pipettes filled with a solution containing (in mmol/L): Cs-aspartate 130, NMDG 10, TEA-Cl 20, Tris-ATP 2.5, Tris-GTP 0.05, $MgCl_2$ 1, EGTA 10, pH 7.2 were used in whole cell voltage clamp experiments. All myocytes were dialyzed with this solution and perfused with normal physiological salt solution for 10 minutes before experiments were initiated. After the initial dialysis period myocytes were bathed with a Na and K free bath solution containing (in mmol/L): NMDG 150, $CaCl_2$ 2, CsCl 5.4, $MgCl_2$ 1.2, Glucose 10, HEPES 5, 4-AP 2 (pH=7.4). All experiments were performed in Na and K free (in and out) solutions

so that Ca^{2+} currents were measured with minimal contamination from overlapping ionic currents. Precautions were made to ensure that leak current was never greater than 0.14 pA. Membrane potential and whole cell currents were measured with standard techniques described in detail previously (Piacentino et al., 2002). Junction potential were not corrected and were less than 10 mV. The cell capacitance was measured using small 10 mV hyperpolarizing test steps from -80 mV to80 mV. Membrane potentials were controlled with an Axopatch 2B (Axon Instrument) voltage-clamp amplifier using pClamp8 (Axon Instrument) software and acquired with a Digidata 1200 analog to digital converter (Axon Instruments). The data were analyzed with Clampfit (Axon Instruments) and presented with Origin 6.0 (Microcal Software, Inc.). I-V relationships were determined under control conditions, with 300 nM, and 10 μM ATI22-107.

Force-Frequency Studies in Isolated Trabeculae. Right Ventricular Trabeculae were isolated from Feline hearts, mounted in a custom made force transducer apparatus, and allowed to equilibrate as previously described(Rossman et al., 2004). Once stabilized (at 0.5 Hz), trabeculae underwent control force-frequency experiments. Steady state twitches were recorded at 0.5, 1.0, 1.5, 2.0, and 2.5 Hz. After control measurements, trabeculae were randomly chosen to be exposed to step doses of Enoximone (300nM, 1uM, 3μM, 10 μM), or ATI22-107 (0.3 μM, 1uM, 3μM,10 μM). A force-frequency experiment was performed at each dose of either Enoximone or ATI22-107. After each dose change or change in stimulation frequency (0.5 to 2.5 Hz), equilibration was allowed until a steady state of force development was achieved.

Statistical Analysis. Results are presented as the mean±SEM unless otherwise stated. Statistical significance was determined by one-way, two-way, or repeated measures two way ANOVA, and a Student-Newman-Keuls post hoc test (Graph Pad Instat and SPSS statistical software). Values of $P<0.05$ were considered significant.

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Results

PDEIII and LTCC Inhibition Assays. As seen in Table 1, ATI22-107 exhibits strong dose dependent inhibition of both PDEIII and LTCC specific activity.

$[Ca^{2+}]_i$ Transients in Isolated Myocytes. Fig. 1A and B show typical Ca^{2+} transient traces of dose-response experiments for both Enoximone and ATI22-107. Fig. 1C.-1F. show that Enoximone induced dose-dependent increases in peak $[Ca^{2+}]_i$, diastolic $[Ca^{2+}]_i$, T_{50}, and T_{75}. ATI22-107 demonstrated similar dose-dependent increases in peak $[Ca^{2+}]_i$ at 300 nM and 1.0 uM doses with no further increases at higher doses. Moreover, throughout the dosing range, ATI22-107 induced little, if any, increase in diastolic $[Ca^{2+}]_i$, T50, and T75 compared to Enoximone. [ATI22-107 n=19, Enoximone n= 8]

$I_{Ca,L}$ Measurements. As illustrated in Fig. 2., ATI22-107 induced dose-dependent decreases in $I_{Ca,L}$ across a physiologic voltage range when compared to baseline. These data demonstrate the activity of the LTCC moiety of the dual-pharmacophore, and indicates that the enhanced peak $[Ca^{2+}]_i$, induced by ATI22-107 can only be attributed to the PDE inhibition moiety of the dual-pharmacophore. [ATI22-107 n=3]

Force-Frequency Studies. Fig. 3 (A-C) depict force transients from isolated trabeculae during increments in stimulation frequency under control conditions and with high dose Enoximone and ATI22-107. Data from all experiments is summarized in Table 1 and Fig. 3 (D, E). Under control conditions, increasing stimulation frequency induces increased developed force (a positive-frequency response), faster rates of force development and force decline, and minimal changes in diastolic force. Enoximone

induced dose-dependent increases in the developed tension and +dF/dt at each frequency. However, Enoximone did not significantly enhance rate-dependent increases in the rate of force decline (-dF/dt). ATI22-107 induced similar dose-dependent increases in developed force and +dF/dt at lower stimulation frequencies (0.5, 1.0, and 1.5 Hz). However, in contrast to Enoximone, ATI22-107 did not augment these parameters at higher stimulation frequencies (2.0 and 2.5 Hz). Another distinction between ATI22-107 and Enoximone is that ATI22-107 produced dose-dependent increases in –dF/dt versus control at lower stimulation frequencies.

Fig. 4. depicts the force-frequency responses as percent change in developed tension vs. 0.5 Hz of the same dose and drug. This depiction of the data clearly demonstrates that the normal positive force-frequency response is preserved by both high and low doses of Enoximone with little change in the overall slope (gain) of frequency-dependent inotropic responses. In contrast, a moderate (1 μM) dose of ATI22-107 tends to reduce the gain of frequency-dependent inotropic responses and a high (10 μM) dose of ATI22-107 prevents increases in developed force beyond 1.5 Hz. [ATI22-107 n=7, Enoximone n= 5]

Discussion

The primary new findings of these studies is that the dual-pharmacophore, ATI22-107, provides a positive inotropic effect that is limited by the increasing contribution of the LTCC -inhibiting moiety at the higher doses. The effect of the LTCC -inhibiting moiety is seen not only in the effects of the compound on the $I_{Ca,L}$ in voltage clamp experiments, but also in whole cell $[Ca^{2+}]_i$ transients from isolated myocytes and the dynamic physiological responses of isolated cardiac trabeculae under physiological loading conditions. At low doses, the effects of ATI22-107 on peak $[Ca^{2+}]_i$ were equivalent to those observed with Enoximone, a pure PDE-III inhibitor, but further increases in peak $[Ca^{2+}]_i$ were not observed at higher doses. Importantly, ATI22-107 induced dose-dependent increases in the decay rate of the $[Ca^{2+}]_i$ transients, as evidenced by the decreasing T_{50} and T_{75} seen in the myocyte experiments, and did not induce the increases in diastolic $[Ca^{2+}]_i$ observed with Enoximone. In trabeculae, increases in –dF/dt with ATI22-107 are also consistent with improved diastolic function. Overall, the combination of PDE-III and LTCC-inhibiting moieties in a single compound has produced a novel pharmacological profile that demonstrates the effectiveness of the dual-pharmacophore technology and may provide a new therapeutic modality for the treatment of heart failure.

The in vitro physiological responses to ATI22-107 clearly reflect the functional activities of each of the chemical moieties incorporated in this dual-pharmacophore. At low doses of ATI22-107 (300 nM and 1 μM), the increases in peak cytosolic calcium in myocytes and increases in developed force in isolated trabeculae were virtually identical to those observed with Enoximone. However, at both low and high doses of the two compounds, distinctions emerged between Enoximone and ATI22-107 that are consistent

with the additional activity of the LTCC inhibitor moiety. Specifically, differences in diastolic $[Ca^{2+}]_i$ levels, $T_{50,}$ and T_{75} are apparent even at 300 nM, and studies using voltage clamp techniques confirm the modulation $I_{Ca,L}$ at this dose. Moreover, doses of ATI22-107 above 1 μM did not produce further increases in peak $[Ca^{2+}]_i$ in isolated myocytes. Finally, differences in modulation of the force-frequency responses by Enoximone and ATI22-107 are also functionally important distinctions between the two compounds. Specifically, because a positive force-frequency response depends on progressive increases in Ca^{2+} entry via the LTCC, the divergence of frequency-dependent responses between ATI22-107 and Enoximone attests to the activity of the LTCC-inhibiting moiety in the dual-pharmacophore under dynamic physiological conditions.

Positive inotropes have been long considered an intuitive and tempting pharmacologic approach for treatment of heart failure. Although existing inotropic agents improve hemodynamic parameters and relieve symptoms among patients with advanced heart failure, these agents have typically been associated with increased mortality rates (Thackray et al., 2002; Klein et al., 2003; Rapezzi et al., 2003; Southworth, 2003). One putative mechanism contributing to increased mortality with existing inotropes is increased diastolic $[Ca^{2+}]_i$ levels which may impair diastolic relaxation and predispose to malignant arrhythmias. In a previous study, ATI22-107 has been successfully shown to improve calcium homeostasis and provide anti-arrhythmic effects in a pacing induced heart failure model (Mazhari, 2004). Our results showing the ability of ATI22-107 to provide limited inotropy, improve diastolic relaxation, and maintain stable diastolic calcium levels is a novel and favorable pharmacological profile that is a likely mechanism for improvement of function in heart failure models, and might provide clinical utility.

Limitations In our myocyte studies, we did not include simultaneous length change with the Ca^{2+} profile data. Although myocyte inotropy and lusitropy as evidenced by myocyte fractional shortening usually corresponds well with the $[Ca^{2+}]_i$ profile, we cannot entirely exclude the possibility that changes in myofilament Ca^{2+} sensitivity altered the relationship between $[Ca^{2+}]_i$ and myocyte shortening and contributed to increases in relaxation rates with ATI22-107. However, the general concordance between our myocyte $[Ca^{2+}]_i$ data and the force measurements in isolated trabeculae support the assertion that increases in peak $[Ca^{2+}]_i$ and rates of decline result in improved contractility and relaxation rates, respectively. We did not provide data examining LTCC inhibitors alone or their comparison with our compound. However, the effects of LTCC inhibitors on normal myocytes and trabeculae are well-established by previously published reports. (Kass, 1982; Leonard and Talbert, 1982; Lindemann et al., 1982; Kanaya et al., 1983; Katz, 1986)

Conclusions To our knowledge, this is the first demonstration of complementary effects of two distinct moieties in a dual-pharmacophore designed for direct modulation of cardiac function. As such, our findings provide a proof of principle for the application of dual-pharmacophore technology to produce specifically targeted therapeutics exhibiting the features of two or more well-characterized compounds. At the same time, the ability of ATI22-107 to enhance inotropy and lusitropy without increasing diastolic $[Ca^{2+}]_i$ levels potentially allows enhancement of function without increasing the risks of arrhthymogenesis and/or ischemia. Due to the growing heart failure epidemic, including the increasing prevalence of patients with advanced symptoms despite application of optimized therapy with vasodilators and β-blockers, the development of a safe and effective inotropic agent would provide a welcome addition to the heart failure therapeutic

armamentarium. Further studies exploring the effects of this and other novel inotropes in failing hearts will be necessary to extend and complement the present investigations.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Dose Dependent Ca2+ Transient Relationship (Normalized and Raw Traces are shown) for A) Enoximone and B) ATI22-107. Enoximone exhibited a dose dependent increase in inotropy throughout the dose range. ATI22-107 followed a similar trend consistently for only the first two doses. The higher doses caused no increase or diminished inotropy. Raw traces clearly show the much larger increases in diastolic calcium by Enoximone when compared to ATI22-107.

Comparisons between Enoximone and ATI22-107 for C) Peak Ca^{2+} , D) Diastolic Ca^{2+}, E) T50, F) T75 (T50, T75 = Time to 50 and 75% descension of the calcium transient respectively.) have been made. Enoximone induced dose-dependent increases in peak $[Ca^{2+}]_i$, diastolic$[Ca^{2+}]_i$, T50, and T75. ATI22-107 demonstrated similar dose-dependent increases at 300 nM and 1.0 uM doses with less or no further increases at higher doses. Throughout the dosing range, ATI22-107 induced much smaller, if any, increases in diastolic $[Ca^{2+}]_i$, T50, and T75. This is indicative of an ability to increase inotropy while maintaining a relatively low $[Ca^{2+}]_i$. * - vs. Enoximone at the equivalent dose ($p<0.05$)

Fig. 2. Dose dependent Ca^{2+} current-voltage relationship for 0, 300 nM, and 10 μM concentrations. A) Representative currents are shown for 0, 300 nM, and 10 μM concentrations of ATI22-107. Notice there is a noticeable decrease in calcium current with increasing dose of ATI22-107 especially at 10 and 20 mV step voltages. B) Current voltage relationship at each dose was found to be statistically different from one another ($p< 0.001$). There is a significant dose dependent decrease in the current across a

physiologic voltage range with increasing doses of ATI22-107. ATI22-107 clearly blocks the LTCC which is most likely responsible for the unchanging diastolic Ca^{2+} levels across all doses and the diminished peak Ca^{2+} at higher doses.

Fig. 3. Effects of ATI22-107 on Feline Trabeculae

Representative traces of raw data from a force frequency experiment in normal feline cardiac trabeculae at A) Control, B) High Dose 10 μM Enoximone and C) High Dose 10 μM ATI22-107. Peak developed force is plotted against frequency separately for each dose of ATI22-107 (D) and Enoximone (E). The force frequency relationship at each dose was found to be statistically different from one another ($p< 0.05$). There appeared to be a positive force-frequency relationship up to 2.0 Hz for all doses except the highest dose. The highest dose (10μM) of ATI22-107 showed a decrease in developed tension at the two higher frequencies (2.0 and 2.5 Hz).

- $p<0.05$ vs. control at same frequency

┼ - $p<0.05$ vs. previous dose at same frequency

* - $p<0.05$ vs. same dose of Enoximone at same frequency

Fig. 4. % Change of Developed Tension compared to 0.5 hz of same drug and dose

This depiction of the developed tension data clearly delineates the divergence of ATI22-107 and Enoximone in generating force with increasing frequency and dose, and thus the role of the LTCC blocking moiety of ATI22-107.

- $p<0.05$ vs. control at same frequency

* - $p<0.05$ vs. same dose of Enoximone at same frequency

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Table 1. Results of PDEIII and LTCC Specific Binding Assays

ATI22-107(Concentration)	PDEIII Activitiy(%)	LTCC Specific Binding(%)
1nM	84.1	100.99
10nM	28.2	101.34
100nM	8.5	78.33
1uM	5.4	32.65
10uM	4.6	18.78

ATI22-107 clearly exhibits dose dependent inhibition of both PDEIII and LTCC activity in biochemical assays. PDEIII activity inhibition was measured by observing the hydrolysis of cAMP. LTCC activity inhibition was measured by liquid scintillation spectrophotometry utilizing the displacement of [3H](+)PN 200-100 by ATI22-107(New England Nuclear,Boston,Mass.).

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Table 2. Trabeculae Force Transient Properties of Dose Dependent Exposure to ATI22-107 and Enoximone at different Frequencies of Stimulation.

N=11	Freq	DevF ± SEM	+dF/dt ± SEM	DiaF ± SEM	-dF/dt ± SEM
	(Hz)	mN / mm2	mN / s / mm2	mN / mm2	mN / s / mm2
ATI 2	0.5	11.6 ± 1.7 #	114.0 ± 19.2 # *	4.1 ± 0.2 # *	-107.5 ± 40.7 *
ATI 4	0.5	14.6 ± 1.3 #+^	183.0 ± 24.8 #+^	4.2 ± 0.2 #	-203.1 ± 49.3 #+^
Control	0.5	9.4 ± 0.4	70.3 ± 8.5	7.2 ± 2.5	-59.7 ± 31.0
ENOX 2	0.5	10.9 ± 0.4 #	85.1 ± 15.7 #	4.9 ± 0.2 #	-47.8 ± 8.9
ENOX 4	0.5	12.5 ± 0.3 #+	105.7 ± 8.2 #+	3.2 ± 0.3 #+	-83.6 ± 12.7 +
ATI 2	1.0	13.6 ± 1.0 #	154.1 ± 20.5 #	3.6 ± 0.2 #*	-143.4 ± 40.8 *
ATI 4	1.0	17.2 ± 1.4 #+^	200.4 ± 30.0 #^	4.3 ± 0.4 #	-200.0 ± 52.0 #+
Control	1.0	11.2 ± 0.8	83.7 ± 11.7	5.9 ± 0.4	-119.5 ± 45.0
ENOX 2	1.0	14.1 ± 1.6 #	144.7 ± 16.0 #	5.1 ± 0.1 #	-71.7 ± 25.9
ENOX 4	1.0	17.6 ± 0.5 #+	144.6 ± 7.6 #	3.1 ± 0.4 #+	-191.2 ± 12.7 #+
ATI 2	1.5	21.0 ± 1.5 #	252.0 ± 35.6 #*	3.8 ± 0.2 *	-191.2 ± 52.6
ATI 4	1.5	23.7 ± 1.8 #	278.2 ± 48.0 #	4.2 ± 0.3 #	-202.8 ± 58.3 #
Control	1.5	19.1 ± 1.2	159.2 ± 16.0	6.6 ± 1.8	-155.3 ± 31.8
ENOX 2	1.5	21.6 ± 1.1 #	230.0 ± 8.3 #	5.0 ± 0.3	-215.1 ± 28.9
ENOX 4	1.5	26.4 ± 1.5 #+	290.0 ± 18.5 #+	3.6 ± 0.1 #+	-239.0 ± 30.9 #
ATI 2	2.0	23.8 ± 1.6 *	292.7 ± 45.3	3.2 ± 0.2 #	-332.1 ± 59.5 #*
ATI 4	2.0	22.2 ± 1.7 *	324.4 ± 55.5	3.9 ± 0.4 #	-228.8 ± 58.4 + *
Control	2.0	21.9 ± 1.2	217.7 ± 22.9	5.6 ± 0.8	-209.1 ± 33.2
ENOX 2	2.0	25.6 ± 1.5 #	252.4 ± 16.9	3.7 ± 0.3	-239.0 ± 26.6
ENOX 4	2.0	27.0 ± 2.5 #	334.0 ± 32.1 #+	3.9 ± 0.1 #	-322.6 ± 46.5 +
ATI 2	2.5	24.6 ± 2.1 *	321.8 ± 47.0 *	4.0 ± 0.2 #	-345.5 ± 58.5 # *
ATI 4	2.5	21.5 ± 1.8 *	326.9 ± 59.4	4.4 ± 0.2 #	-281.9 ± 63.1 +
Control	2.5	23.1 ± 1.4	257.5 ± 28.4	5.2 ± 1.0	-266.8 ± 36.3
ENOX 2	2.5	24.0 ± 2.3	257.4 ± 29.9	3.9 ± 0.6 #	-250.9 ± 27.2
ENOX 4	2.5	29.3 ± 2.2 #+	337.0 ± 20.8 #	3.5 ± 0.1	-301.4 ± 34.7

MEAN ± SEM
- p<0.05 vs. control at same frequency
+ - p<0.05 vs. previous dose at same frequency
^ - p<0.05 vs. same dose of Enoximone at same frequency

ATI - ATI22-107
ENOX - ENOXIMONE
2 - 1 μM
4 - 10 μM

DevF - Developed Force
DiaF - Diastolic Force

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Fig. 1.

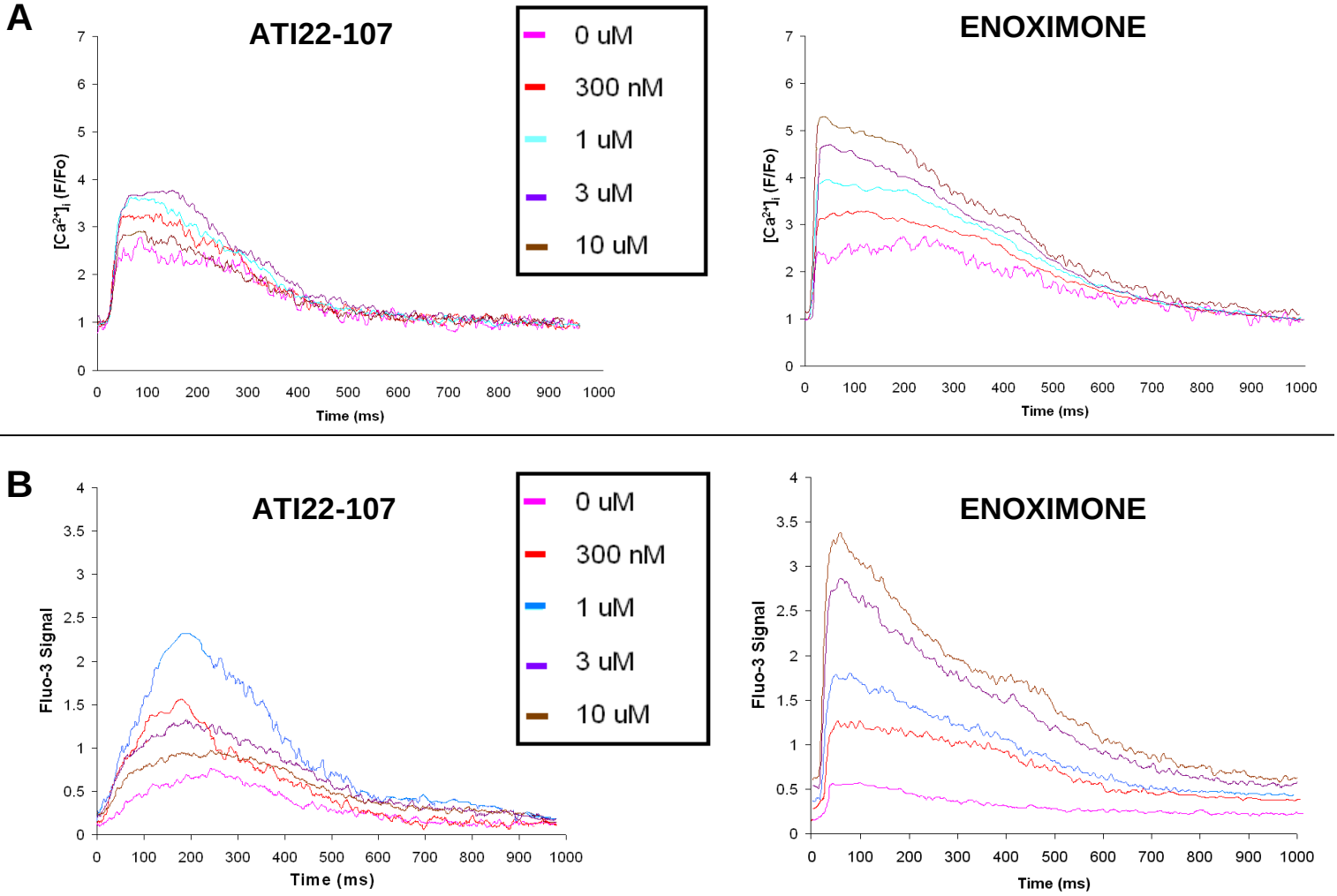

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Fig. 2.

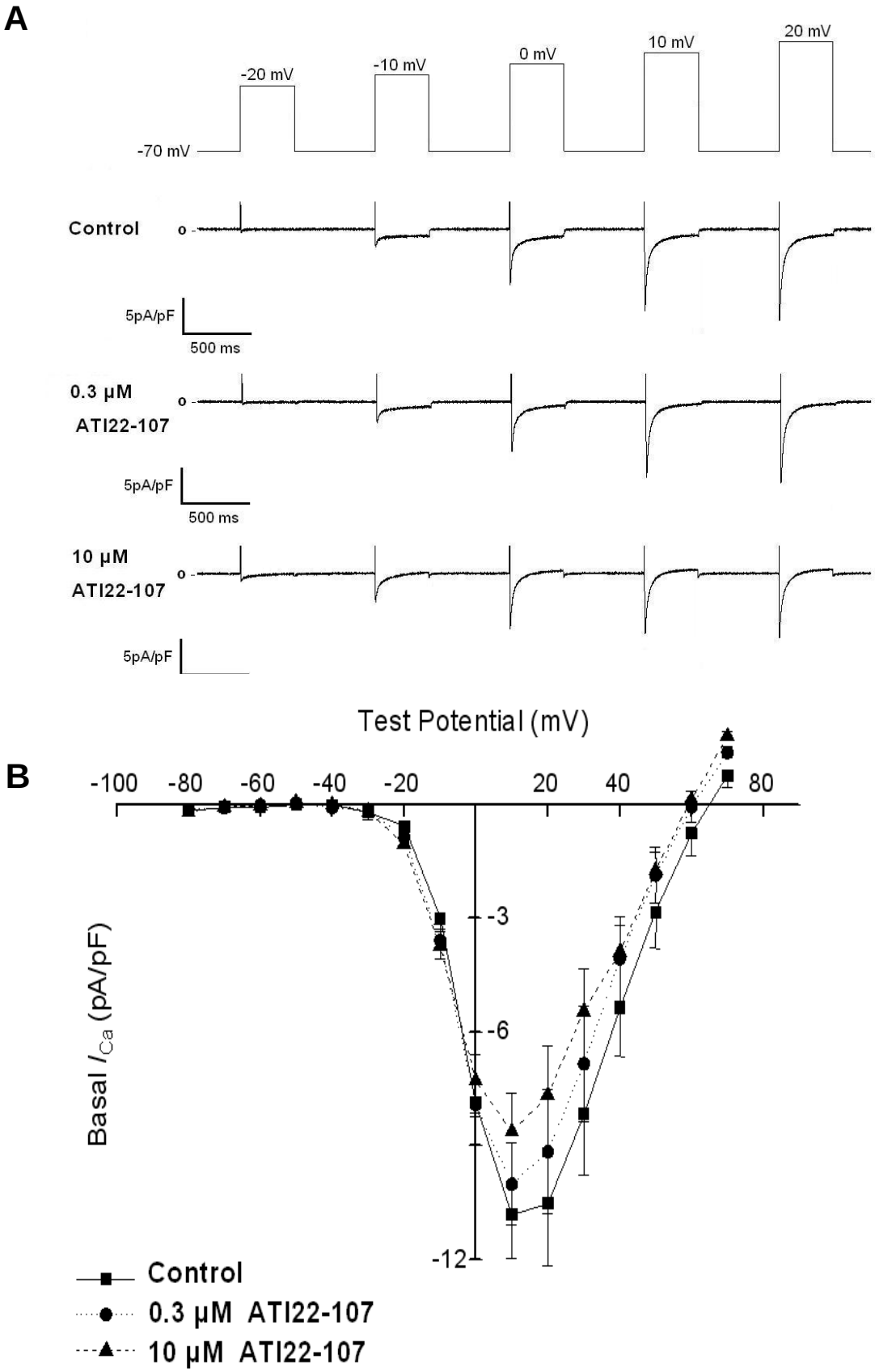

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Fig. 3.

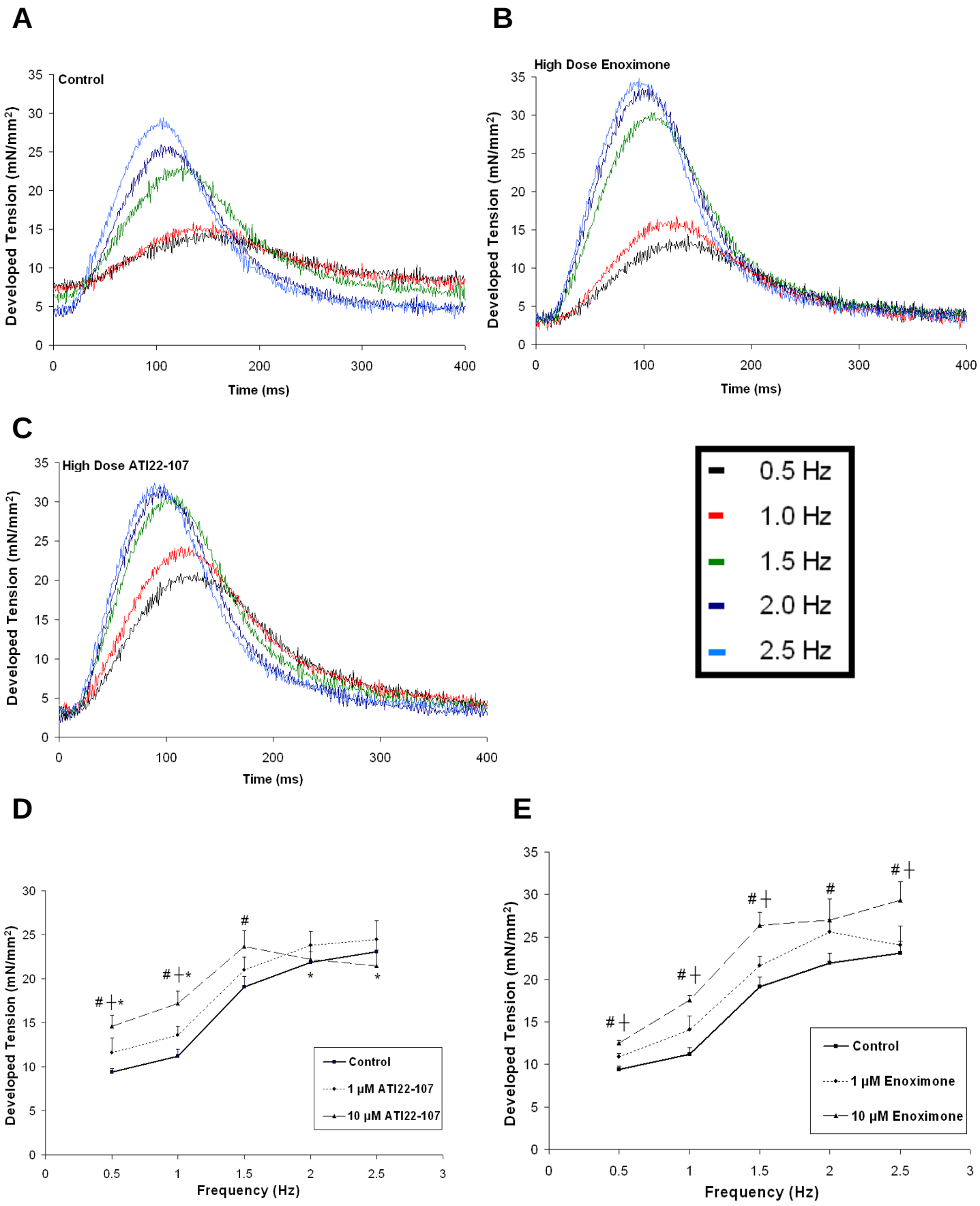

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Fig. 4.