A Highly Specific Benzimidazole Pyridazinone Reverses Phosphate-Induced Changes in Cardiac Myofilament Activation†

Margaret V. Westfall, Gordon M. Wahler, and R. John Solaro*

Department of Physiology and Biophysics, University of Illinois—Chicago, Chicago, Illinois 60612

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ABSTRACT: Both Ca^{2+} and the actin cross-bridge reaction itself can activate contraction in myofilaments. We are interested in identifying ligands which modify one or both mechanisms of contractile activation with high affinity and specificity. Results presented here suggest that the benzimidazole-substituted pyridazinone, UD-CG 212 Cl, potently modulates myofilament activation by the cross-bridge reaction. Cross-bridge-mediated activation was studied by varying the population of force-generating (strong) cross-bridges with inorganic phosphate (P_i). Addition of P_i to detergent-extracted (skinned) canine vetricular preparations reduces the population of strong cross-bridges, which causes reduced myofilament force and Ca^{2+} sensitivity. Increased P_i concentration ($[P_i]$) also favors cross-bridge-mediated myofilament activation, so cooperativity increases. The decreased Ca^{2+} sensitivity produced by P_i was reversed by a racemic mixture of 10^{-10} M UD-CG 212 Cl, but this agent had no effect on maximum force or cooperativity. In experiments with stereoisomers, only (+)-UD-CG 212 Cl stimulated force. At higher doses (10^{-6}–10^{-4} M), submaximal but not maximal force decreased and (−)-UD-CG 212 Cl was the active stereoisomer. Neither P_i nor UD-CG 212 Cl affected Ca^{2+} binding to myofilament troponin C (TnC). Thus, UD-CG 212 Cl appears to reverse P_i-induced decreases in submaximal force via high-affinity binding to a myofibrillar domain not directly involved with myofibrillar TnC–Ca^{2+} binding. The actions of UD-CG 212 Cl were further investigated by reducing [ATP] as another means of varying the cross-bridge population. The increase in submaximal, Ca^{2+}-activated force observed with a moderate reduction in [ATP] was further increased by UD-CG 212 Cl. These results support our hypothesis that UD-CG 212 Cl modulates strong cross-bridge activation of the thin filament.

Striated myofilament activation is complex and depends on Ca^{2+} binding to troponin C (TnC)[1] as well as the population of actin-attached, force-generating (strong) cross-bridges (Figure 1A). Studies of Ca^{2+}-mediated myofilament activation indicate Ca^{2+} binding to TnC initiates protein–protein interactions within the myofilament functional unit (myosin/actin/tropomyosin/troponin in a 1:7:1:1 ratio) (Brandt *et al.*, 1987). These interactions promote force generation by removing the inhibitory influence of tropomyosin (Tm), such that strong attachments form between the myosin cross-bridge and actin [for review see Ford (1991)]. There is also compelling evidence for the idea that strong cross-bridges themselves spread activation by disinhibiting neighboring thin filaments (Bremel & Weber, 1972; Ford, 1992; Millar & Homsher, 1990). However, the mechanism of strong cross-bridge activation of the thin filament is not completely understood.

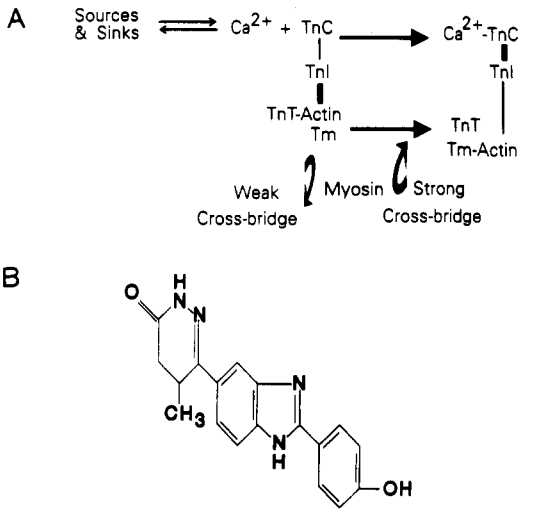

FIGURE 1: (A) Schematic diagram of Ca^{2+}- and strong cross-bridge-mediated activation of cardiac myofilaments. In this scheme, Ca^{2+} binding to TnC weakens the bond between actin and TnI, which causes Tm to be removed from its blocking position. The cross-bridge is then able to form a strong, force-generating attachment with actin. This scheme also illustrates that similar types of conformational changes are induced by strong cross-bridges on immediately adjacent functional units. (B) Molecular structure of UD-CG 212 Cl.

Our laboratory is interested in using high-affinity ligands to study myofilament activation. The idea behind this approach is based on the successful use of high-affinity ligands and toxins to study the functional properties of other complex systems, such as membrane channels (Janis & Triggle, 1984).

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* Address correspondence to this author at the Department of Physiology and Biophysics, University of Illinois at Chicago, 901 South Wolcott Ave., Room E202, Chicago, IL 60612-7342. Phone: (312) 996-7620; Fax: (312) 996-1414.

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[1] Abbreviations: analysis of variance (ANOVA); dibromobis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (dibromo-BAPTA); dimethyl sulfoxide (DMSO); ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA); high relaxing (HR); Hill coefficient (Hill *n*); 2-[4′-(iodoacetamido)anilino]naphthalenesulfonic acid (IAANS); inorganic phosphate (P_i); low relaxing (LR); −log $[Ca^{2+}]$ required for 50% activation (pCa_{50}); tropomyosin (Tm); troponin C (TnC); cardiac troponin C (cTnC).

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While there are agents which bind to myofilaments and alter Ca^{2+} activation (Fujino *et al.*, 1988), they do so with relatively low affinity and low specificity. Results from studies with these agents indicate that they affect the Ca^{2+}–TnC pathway, but their ability to modulate the spread of activation by strong cross-bridges has not been thoroughly investigated.

In experiments reported here, we have investigated the effects of the benzimidazole-substituted pyridazinone, UD-CG 212 Cl (Figure 1B), on force generation in demembranated fiber preparations from cardiac muscle. Our results show that this drug has unique actions on myofilament force. Subnanomolar concentrations of UD-CG 212 Cl increased submaximal force when cross-bridge state was varied by addition of inorganic phosphate (P_i) or reduction of ATP. This action of UD-CG 212 Cl was stereospecific and occurred with no change in Ca^{2+} binding to the myofilament. We postulate that UD-CG 212 Cl may act at one or more novel, high-affinity site(s) on the myofilament, and thus, it may be a useful probe in understanding myofilament activation.

METHODS

Force Measurements in Skinned Cardiac Preparations from Dog. Trabeculae from the left ventricle of dog hearts were dissected and skinned in 1% Triton X-100 at 4 °C for 12 h as described by Pan and Solaro (1987). Preparations were then transferred to a relaxing solution containing 50% glycerol and stored for up to 3 weeks at −20 °C. Bundles with a diameter less than 0.2 mm (length = 10 mm) were glued to an AME-801 force transducer (SensoNor, Horten, Norway) or a transducer described by Chiu *et al.* (1982) on one end and an aluminum rod connected to a micromanipulator on the other end. Similar results were obtained with both force transducers.

Muscles were initially bathed in "high" relaxing [HR solution; 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)] and then "low" relaxing (LR solution; 0.10 mM EGTA) solutions, both of which contained 2 or 5 mM Mg^{2+}, 5 mM $MgATP^{2-}$, 12 mM creatine phosphate, and 60 mM imidazole (ionic strength = 150 mM, pH = 7.0). The effect of UD-CG 212 Cl on force was the same for the two different Mg^{2+} concentrations (results not shown). The Mg^{2+} concentration used in individual experiments is designated in the figure legends. Mean sarcomere length was measured from the laser diffraction pattern (Hibberd & Jewell, 1982) in HR solution. Isometric force was recorded at room temperature on a chart recorder (Linear Instruments, Irvine, CA). Contraction solutions contained Ca^{2+} added to achieve the desired free [Ca^{2+}] plus the reagents contained in high relaxing solution. The software program BATHE (kindly provided by Dr. Robert Godt), which is based on the equations of Godt and Lindley (1982), was used to determine the Ca^{2+} concentration needed for the desired free Ca^{2+} concentration which is expressed as the pCa (−log molar free Ca^{2+} concentration).

The direct effect of UD-CG 212 Cl on myofilaments was evaluated in the presence and absence of exogenously added P_i (5, 10 mM). The endogenous P_i concentration in our solutions was approximately 1 mM. In most experiments, maximum force was measured before and after every other contraction. However, when the muscle was incubated in several consecutive contracting solutions maximum force was measured before and after every contraction. The effect of UD-CG 212 Cl on force also was examined following the reduction of ATP concentration. UD-CG 212 Cl (kindly supplied by Dr. Karl Thomae Pharmaceuticals, Biberach-an-der-Riss, Germany) was dissolved in dimethyl sulfoxide (DMSO), and diluent (0.5%) was included in all solutions. DMSO (0.5%) alone had no effect on force development (results not shown). The base form of UD-CG 212 was used when the effects of pure stereoisomers were measured. Ionic strength was maintained at 150–165 mM in experiments with added P_i or reduced ATP concentrations.

Ca^{2+} Binding to Skinned Cardiac Preparations. Ca^{2+} binding was measured in unrestrained skinned cardiac preparations as described in detail by Pan and Solaro (1987). Our laboratory previously showed that Ca^{2+} binding to myofilament TnC was not significantly different in restrained and unrestrained fibers (Pan & Solaro, 1987). After preparations were incubated in binding solution for 100 min, $^{45}Ca^{2+}$ and [^{3}H]glucose were eluted from preparations for 12 h at room temperature. Binding solution contained 4 μCi/mL $^{45}Ca^{2+}$ (ICN Biomedicals, Inc., Costa Mesa, CA) and 3–4 μCi/mL [^{3}H]-D-glucose (Dupont NEN, Hoffman Estates, IL). Elution solution and an aliquot of binding solution were counted on a Packard Tri-Carb 2200CA liquid scintillation counter.

Cardiac TnC (cTnC) Experiments. Bovine cTnC was prepared as described by Potter (1982). The effect of UD-CG 212 Cl on cTnC binding of Ca^{2+} was determined in the presence of 50 μM of the Ca^{2+} chelator dibromobis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (dibromo-BAPTA). Other conditions were 90 mM KCl and 10 mM MOPS, pH 7.0. Absorbance changes in response to dibromo-BAPTA chelation of Ca^{2+} were monitored at 260 nm (Tsien, 1980) in the presence and absence of cTnC and/or UD-CG 212 Cl. DMSO (0.5%) and UD-CG 212 Cl (10^{-10} M) did not change dibromo-BAPTA absorbance in the absence of cTnC. In addition, we studied the effect of UD-CG 212 Cl on Ca^{2+} binding to cTnC labeled with 2-[4′-(iodoacetamido)anilino]-naphthalenesulfonic acid (IAANS) as described by Johnson *et al.* (1980).

Statistics. Repeated measurements are expressed as mean ± SEM. Statistical comparisons were analyzed using a one-way analysis of variance (ANOVA) and post-hoc Tukey test unless otherwise noted. Normalized force measurements were fit to the Hill equation using iterative nonlinear regression (Graphpad; ISI software, version 2.0) to derive the pCa_{50} (−log [Ca^{2+}] required for 50% activation of force) and the Hill coefficient (Hill *n*). The pCa_{50} is used as an indicator of myofibrillar Ca^{2+} sensitivity, and the Hill *n* is used as an index of cooperative interactions within the myofibril.

RESULTS

Our pilot studies showed that low doses of UD-CG 212 Cl increased force when the state of myofilament activation was changed. Myofilament state was altered by adding 5 mM P_i, which increases the population of weakly-attached cross-bridges (Ruegg *et al.*, 1971; Millar & Homsher, 1990) and, thus, decreases force (Table I). Figure 2 shows an experiment in which the submaximal force observed in the presence of 5 mM P_i increased significantly upon addition of 10^{-10} M UD-CG 212 Cl. This dose of UD-CG 212 Cl had no effect on submaximal force in the absence of added P_i (Table I). When the P_i concentration was further increased to 10 mM, 10^{-10} M UD-CG 212 Cl tended to increase submaximal force more than the increase observed with 5 mM P_i (Table I).

The relation between UD-CG 212 Cl concentration and the change in submaximal, Ca^{2+}-activated force is shown in Figure 3. Addition of 10^{-14}–10^{-5} M UD-CG 212 Cl at pCa 5.50 produced a biphasic effect on force in the presence of 5 mM P_i. Low concentrations of UD-CG 212 Cl (10^{-10}, 10^{-9}

Table I: Effect of Exogenously Added P_i and 10^{-10} M UD-CG 212 Cl on Force[a]

pCa	added [P_i] (mM)	n	% maximum force[b]	n	% change[c] [(+)-UD-CG 212 Cl]
5.50	0	8	70.2 ± 1.0	4	3.3 ± 1.7
5.50	5	8	43.3 ± 2.4	3	22.4 ± 5.2*
5.50	10	4	35.2 ± 3.1	3	31.5 ± 3.8*
4.50	0	8	100.0 ± 0.0		ND
4.50	5	10	68.7 ± 2.4†	3	0.5 ± 2.7
4.50	10	5	56.2 ± 4.0†,‡	3	0.1 ± 1.6
4.50	20	2	57.3		ND

[a] Values are mean ± SEM, and ND indicates experiment was not done. [b] These values were calculated using force at pCa 4.50 (no added P_i) as maximum force. [c] The effect of UD-CG 212 Cl was determined by calculating the percent change from the corresponding tension observed at the same P_i without UD-CG 212 Cl. UD-CG 212 Cl concentration used for these experiments was 10^{-10} M. Statistical comparisons were carried out using a one-way ANOVA and a Tukey multiple comparison test. *$p < 0.05$ vs pCa 5.50, no added P_i. †$p < 0.05$ vs pCa 4.50, no added P_i. ‡$p < 0.05$ vs pCa 4.50, 5 mM P_i.

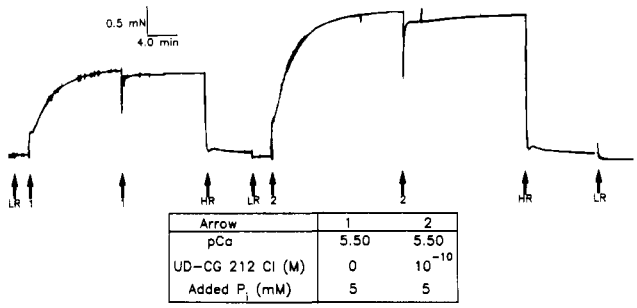


FIGURE 2: Representative tracing of the 10^{-10} M UD-CG 212 Cl effect on force at pCa 5.50 in the presence of 5 mM P_i. Relaxing [high relaxing = 10 mM EGTA (HR) and low relaxing = 0.1 mM EGTA (LR)] and contracting solutions contained 5 mM free Mg^{2+}. Contractions without and then with UD-CG 212 Cl were performed two consecutive times to ensure that changes in force caused by UD-CG 212 Cl were not due to solution changes. In the presence of 5 mM P_i, UD-CG 212 Cl increased force.

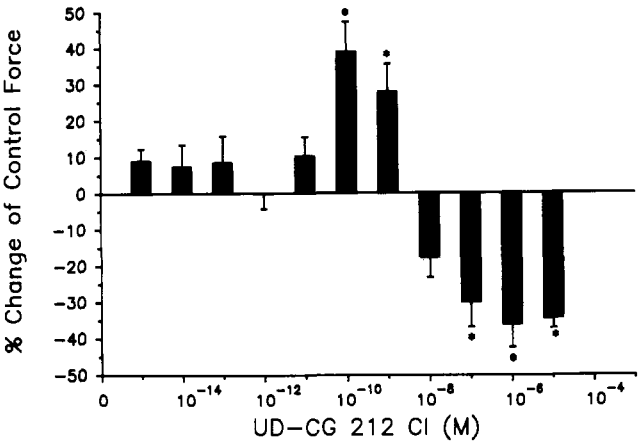


FIGURE 3: Effect of UD-CG 212 Cl concentration of force at pCa 5.50 in the presence of 5 mM P_i. Incubation conditions were the same as those described in Figure 2. Force is expressed as the percent change in force compared to a paired measurement made at pCa 5.50 in the presence of 5 mM P_i. Measurements were made on 3–7 cardiac preparations. *$p < 0.05$ vs no UD-CG 212 Cl using one-way ANOVA and post-hoc Tukey test.

M) significantly increased submaximal force. These low doses of UD-CG 212 Cl had no effect on force in the absence of added P_i (results not shown). Submaximal force decreased with higher concentrations of UD-CG 212 Cl (10^{-6}–10^{-4} M) in the presence (Figure 3) and absence of added P_i (results

Table II: Effect of 5 mM P_i and UD-CG 212 Cl on pCa_{50} and Hill n Values[a]

[UD-CG 212 Cl] (M)	added [P_i] (mM)	n	pCa_{50}	Hill n	r^2
0	0	3–6	5.45 ± 0.01	3.24 ± 0.25	0.994
5×10^{-5}	0	3–6	5.37 ± 0.01*	3.49 ± 0.25	0.995
0	0	4	5.46 ± 0.01	2.76 ± 0.23	0.995
0	5	4	5.39 ± 0.01*	4.38 ± 0.26	0.998
1×10^{-10}	5	4	5.50 ± 0.01†	5.69 ± 0.49*	0.998

[a] Nonlinear regression analysis was used to derive the pCa_{50} and Hill coefficient (Hill n). The values shown are the mean ± SEM, and n indicates the number of different muscle preparations used to derive the pCa_{50} and Hill n. The correlation index (r^2) for each regression analysis is also shown. Results were compared using a Student's unpaired t-test, with $p < 0.05$ considered significant. *$p < 0.05$ vs 0 M UD-CG 212 Cl. †$p < 0.05$ vs 5 mM P_i.

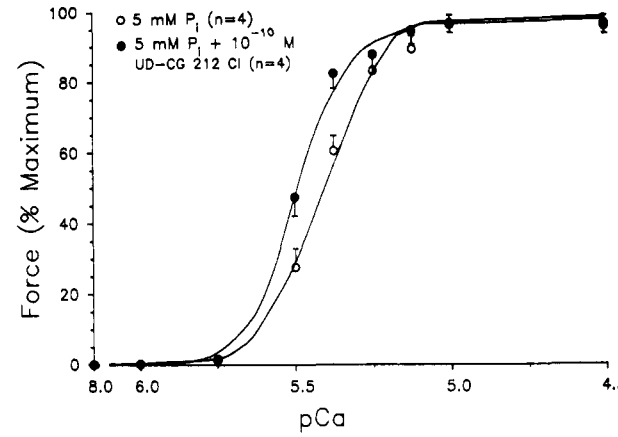


FIGURE 4: Force/pCa relationship in the presence of 5 mM P_i with 10^{-10} M UD-CG 212 Cl. Force measurements were made under the same conditions described in Figure 2. Force was normalized to the corresponding maximum force at pCa 4.50. Addition of P_i decreased maximum force compared to control values (see Table I). The maximum force, pCa_{50}, and Hill n values derived from these curves are shown in Table II.

not shown). Maximal reduction of force by UD-CG 212 Cl could not be determined due to precipitation of the drug at concentrations higher than 150 μM.

Experiments also were done to evaluate the effect of low UD-CG 212 Cl concentrations on the force/pCa relationship. Force measurements made following addition of 5 mM P_i alone agree with numerous earlier reports (Millar & Homsher, 1990; Nosek *et al.*, 1990; Ruegg *et al.*, 1972). Addition of 5 mM P_i reduced maximum force (control = 2.2 ± 0.1 mN, $n = 4$; 5 mM P_i = 1.4 ± 0.1, $n = 4$; $p < 0.05$ vs C using Student's t-test), reduced pCa_{50}, and increased Hill n values (Table II). The reduced pCa_{50} observed with 5 mM P_i was restored to control values with the addition of subnanomolar concentrations (10^{-10} M) of UD-CG 212 Cl (Figure 4, Table II). This low dose of UD-CG 212 Cl had no effect on the P_i-induced increase in Hill n. Low concentrations of UD-CG 212 Cl had no significant effect on maximum force in the presence (Table I) or absence (results not shown) of exogenously added P_i. The specificity of the UD-CG 212 Cl effect on submaximal force was evaluated using the stereoisomers of the drug in the presence of added P_i (Figure 5). Addition of 10^{-10} M (+)-UD-CG 212 Cl increased contractile force, but (–)-UD-CG 212 Cl had no effect on force at this concentration (Figure 5A).

We also performed experiments to determine whether the same stereoisomer decreased submaximal force at higher concentrations of UD-CG 212 Cl. Prior to doing these

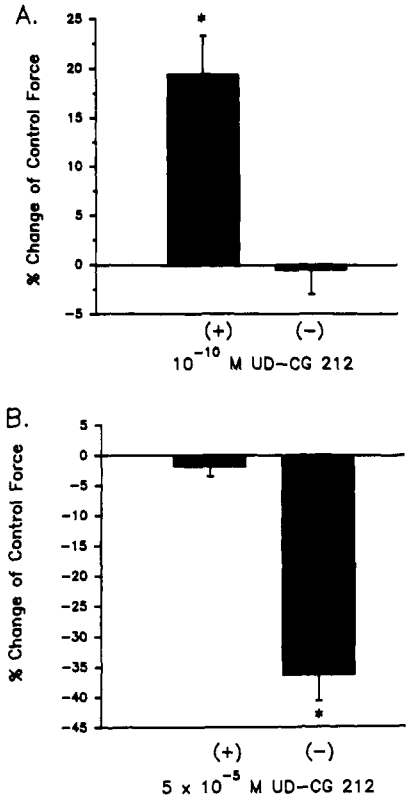


FIGURE 5: Effect of (+)- and (-)-UD-CG 212 at pCa 5.50 on force. (A) Force measurements from 5 experiments in which muscles were exposed to 10^{-10} M (+)- and (-)-UD-CG 212 Cl in the presence of 5 mM P_i. (B) Effect of 5×10^{-5} M (+)- and (-)-UD-CG 212 at pCa 5.50 on force in 5 experiments. All results are expressed as a percentage of force observed at pCa 5.50 ($+P_i$ = 1.0 ± 0.2 mN, n = 5; no added P_i = 2.0 ± 0.5 mN, n = 5). *$p < 0.05$ vs no drug using one-way ANOVA and Tukey multiple comparison test.

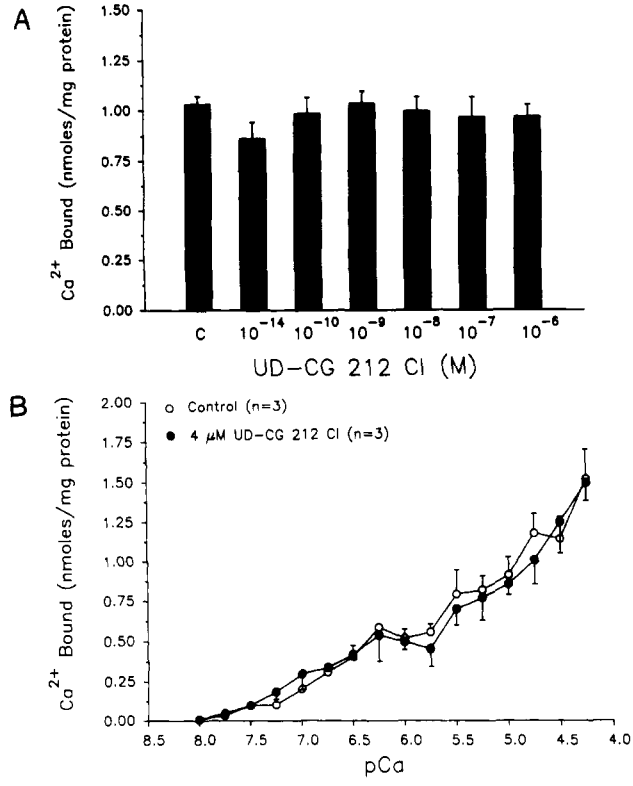


FIGURE 6: UD-CG 212 Cl effect on Ca^{2+} binding to skinned dog cardiac preparations. (A) Ca^{2+} binding at pCa 5.50 with 5 mM P_i in the presence of various concentrations of UD-CG 212 Cl (10^{-14}–10^{-6} M). Measurements were made in the presence of 2 mM Mg^{2+}, but similar results were observed with 5 mM Mg^{2+}. Ca^{2+} binding was not different from control values when results were analyzed with a one-way ANOVA. (B) Ca^{2+} binding over a range of Ca^{2+} concentrations in the presence and absence of 4 μM UD-CG 212 Cl. Ca^{2+} binding was not different with and without UD-CG 212 Cl when results were analyzed with a one-way ANOVA.

experiments, we studied the effect of high UD-CG 212 Cl doses on the force/pCa relationship to ascertain whether maximal force was changing in addition to submaximal force. Table II shows that 5×10^{-5} M UD-CG 212 Cl decreased the pCa_{50} without affecting the Hill n. Maximum force was not affected by this concentration of UD-CG 212 Cl (results not shown). Similar results were previously reported by Bohm *et al.* (1991). The experiments shown were done in the absence of added P_i, but similar findings were obtained with the addition of 5 mM P_i (results not shown). In experiments with stereoisomers, reduced submaximal force was observed with (-)-UD-CG 212 Cl but not the (+) isomer (Figure 5B). Maximal force was decreased slightly by (-)- but not (+)-UD-CG 212 Cl at high concentrations (50 μM; results not shown). These results show opposite stereoselectivity for the stimulation and depression of submaximal force by UD-CG 212 Cl, which indicates that high doses of (-)-UD-CG 212 Cl may cancel the effects of low (+)-UD-CG 212 Cl doses. We postulate from these results that high doses of UD-CG 212 Cl act at different site(s) from the "high-affinity" myofibrillar site(s).

In subsequent experiments, we focused on the possible mechanism(s) causing the force alterations in the presence of low and high UD-CG 212 Cl concentrations. One possible site of UD-CG 212 Cl action may be to alter Ca^{2+} binding to myofilament TnC. UD-CG 212 Cl in a concentration range that increased submaximal force did not alter Ca^{2+} binding to skinned cardiac preparations at pCa 5.50 with 5 mM P_i present (Figure 6A). Ca^{2+} binding to skinned cardiac preparations at this pCa represents half-maximal Ca^{2+} binding to the regulatory site on TnC (Pan & Solaro, 1988). Previous studies have established that an increase in P_i itself does not change Ca^{2+} binding (Takayasu & Solaro, 1990). In the absence of added P_i, Ca^{2+} binding decreased with 5×10^{-5} M but not 10^{-4} M UD-CG 212 Cl at pCa 5.50. We also observed that Ca^{2+} binding between pCa 8.0 and 4.0 was not affected by a higher dose of UD-CG 212 Cl (Figure 6B). There were no increases in Ca^{2+} binding to cTnC in response to 10^{-10} M UD-CG 212 Cl when monitoring changes in BAPTA absorbance as a measure of the partition of Ca between chelator and cTnC (Figure 7). We obtained the same result using $cTnC_{IAANS}$ fluorescence as a reporter of Ca^{2+} binding to the single regulatory site (results not shown). Thus, neither the increased force observed in the presence of low UD-CG 212 Cl concentrations and added P_i nor the decreased force observed with high UD-CG 212 Cl concentrations can be attributed to changes in Ca^{2+} binding to myofibrillar TnC.

Experiments with variable ATP concentrations were carried out to determine whether UD-CG 212 Cl could affect force when the strong/weak cross-bridge ratio was varied without using P_i. Our results with ATP alone agree with those of previous investigators (Brandt *et al.*, 1990; Bremel & Weber, 1972; Godt, 1974). Reduction of ATP to 1 μM resulted in near-maximal force at pCa 8.0 (Figure 8A). Addition of UD-CG 212 Cl had no effect on force under these conditions. In the presence of 20 μM ATP, force was increased at pCa 6.50 and 5.50 compared to control values (5 mM ATP). UD-CG 212 Cl increased submaximal force in the presence of 20

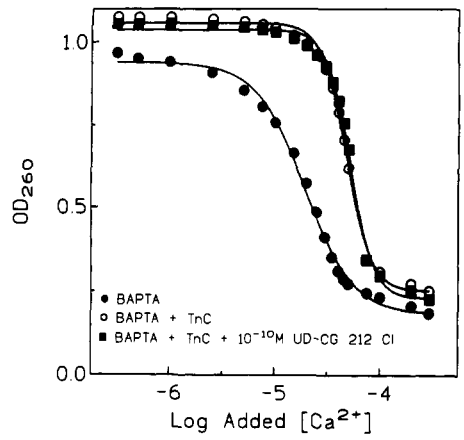
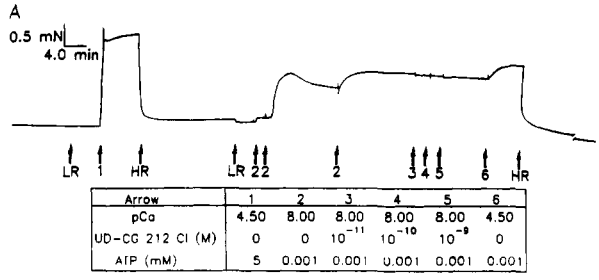
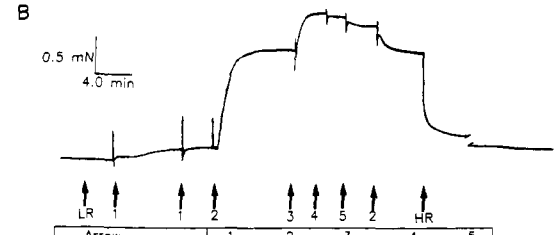


FIGURE 7: Lack of effect of UD-CG 212 Cl on BAPTA absorbance in the presence of TnC. The decrease in BAPTA absorbance with increasing Ca^{2+} concentrations is shifted to the right when TnC is added and Ca^{2+} binds to TnC. Addition of 10^{-10} M UD-CG 212 Cl did not further shift the curve observed with TnC and BAPTA.



Arrow	1	2	3	4	5	6
pCa	4.50	8.00	8.00	8.00	8.00	4.50
UD-CG 212 Cl (M)	0	0	10^{-11}	10^{-10}	10^{-9}	0
ATP (mM)	5	0.001	0.001	0.001	0.001	0.001



Arrow	1	2	3	4	5
pCa	8.0	5.50	5.50	5.50	5.50
UD-CG 212 Cl (M)	0	0	10^{-11}	10^{-10}	10^{-9}
ATP (mM)	0.02	0.02	0.02	0.02	0.02

FIGURE 8: Effect of UD-CG 212 Cl on force measured in the presence of low ATP concentration. (A) Representative recording of force measured in the presence of 1 μM ATP at pCa 8.0 and 4.50. The effect of UD-CG 212 Cl (10^{-11}–10^{-9} M) also was examined in the preparation and produced minimal changes in force. Maximum force in the presence of 5 mM ATP also is shown. (B) Representative recording of submaximal force measured at pCa 5.50 with 20 μM ATP in the presence and absence of 10^{-11}–10^{-9} M UD-CG 212 Cl. Measurements were made using 2 mM free Mg^{2+}.

Table III: Effect of UD-CG 212 Cl on Force at pCa 6.50 with 20 μM ATP[a]

[UD-CG 212 Cl] (M)	n	% change from control
10^{-11}	3	17.2 ± 12.0
10^{-10}	3	37.1 ± 11.5*
10^{-9}	3	30.5 ± 6.2*

[a] Values are mean ● SEM. Results were compared to force at pCa 6.50 without UD-CG 212 Cl. Statistical analyses were done using a one-way ANOVA and a Tukey multiple comparison test with $p < 0.05$ (*) considered significant.

μM ATP (Figure 8B, Table III), but had no effect on maximal force (results not shown).

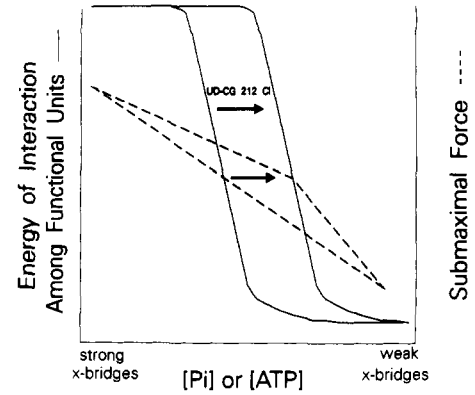


FIGURE 9: A schematic representation of the effects of P_i and ATP concentration on force and functional unit interactions. The left-hand curves are representative of changes that occur in force and functional unit interaction energy with increasing P_i or ATP concentrations. Our working hypothesis of UD-CG 212 Cl effects on force and on interactions between functional units also is shown (right-hand curves). The arrow indicates the rightward shift in force and interaction energy caused by low concentrations of UD-CG 212 Cl as P_i and ATP concentrations increase.

DISCUSSION

Our most important findings are that (1) UD-CG 212 Cl increases submaximal force at subnanomolar concentrations (Figures 3 and 4) in a stereoselective manner (Figure 5) and (2) this action is promoted when the proportion of strong cross-bridges is relatively low. These results indicate tight and, presumably, stereoselective binding of UD-CG 212 Cl to receptor-like domain(s) on the myofilaments. These properties of UD-CG 212 Cl are unique in comparison to other agents which act on the myofilaments. We postulate that UD-CG 212 Cl affects the cross-bridge–thin filament interaction at sites involved in myofilament activation.

The stimulating actions of UD-CG 212 Cl on cardiac myofilaments are dependent on the concentration of P_i surrounding the preparations. Thus, we will first discuss the known actions of P_i, which should provide insight into the site(s) of UD-CG 212 Cl action on the myofilament. Elevated P_i decreases maximum force (Table I), increases pCa_{50}, and increases cooperativity, as reflected in the Hill n (Table II).

Addition of P_i to skinned muscle works to decrease maximal force by shifting the steady state in favor of a population of weak cross-bridges (Millar & Homsher, 1990; Walker *et al.*, 1992). UD-CG 212 Cl has no significant effect on maximum force in the presence of P_i (Table I).

The increase in the weakly attached cross-bridge population produced by elevated P_i also contributes to the decreased myofibrillar Ca^{2+} sensitivity ($\downarrow pCa_{50}$). This shift in cross-bridge population alters the number of nearest-neighbor interactions between functional units, which we have represented schematically in Figure 9. In the absence of added P_i, a high proportion of cross-bridges are force-generating and an optimal number of interactions occur between functional units at any given Ca^{2+} concentration (Millar & Homsher, 1990). As a result, submaximal force and myofilament Ca^{2+} sensitivity are relatively high (Table II). An increase in P_i concentration decreases the population of strong, force-generating cross-bridges, which results in fewer neighboring strong cross-bridges and a corresponding reduction in submaximal force (Figure 9) and Ca^{2+} sensitivity (Millar & Homsher, 1990; Walker *et al.*, 1992). Weakly attached cross-bridges predominate with elevated P_i concentrations. Submaximal force is low and Ca^{2+} sensitivity (Table II) is decreased in this situation, because there are fewer strong

cross-bridges reacting, thereby activating fewer neighboring functional units (Figure 9).

The mechanism whereby the strong cross-bridges activate the thin filament is not completely understood. However, results from our laboratory have shown that increased P_i and the associated decrease in strong cross-bridge population do not affect Ca^{2+} binding to myofilament TnC (Takayasu & Solaro, 1990). It is apparent, therefore, that the strong cross-bridges depicted in Figure 1A influence steps beyond Ca^{2+} binding to TnC.

The mechanism by which UD-CG 212 Cl reverses the effects of P_i on Ca^{2+} sensitivity (Table II, Figure 4) can be partially elucidated from the known actions of P_i. UD-CG 212 Cl could directly increase the number of strong cross-bridges or increase the force generated by a strong cross-bridge. We think these possibilities are unlikely because the drug had little effect on maximum force. UD-CG 212 Cl might also reverse the P_i effect on pCa_{50} by activating the thin filament in a process independent of cross-bridge interactions. However, we do not favor this possibility because UD-CG 212 Cl had no effect on the force/pCa relationship at low P_i concentrations (Table I). A third mechanism of action that seems possible is that the drug affected thin filament activation by strong cross-bridges. We favor this idea on the basis of our results with UD-CG 212 Cl in the presence of P_i (Figure 4), as well as the effect of drug with varying ATP concentrations (Figure 8 and Table III, discussed below). It is also possible that UD-CG 212 Cl acts directly on the cross-bridge to produce states analogous to those induced by elevated MgADP. Like addition of UD-CG 212 Cl, Lu *et al.* (1992) recently showed that addition of MgADP had little effect on maximum force but increased the Ca^{2+} sensitivity of rabbit psoas fibers.

Our results can be interpreted using a theoretical relation between the population of strong cross-bridges and the interaction energy among adjacent functional units which we detected as a change in force as shown in Figure 9. On the basis of our hypothesis, UD-CG 212 Cl would have little effect on force at low P_i levels because strong cross-bridges predominate and relatively few adjacent functional units are available for activation by neighboring cross-bridges. As P_i concentrations increase, fewer strong cross-bridges are present. Under this condition, addition of UD-CG 212 Cl could stimulate submaximal force by increasing the likelihood that neighboring functional units are activated. The enhanced interaction among adjacent functional units produced by UD-CG 212 Cl, in the presence of elevated P_i levels, is depicted in Figure 9 as a rightward shift in the function relating the energy of interaction to cross-bridge population. Our results with low and moderately increased P_i concentrations are in agreement with this prediction (Tables I and II, Figure 4). At very high P_i concentrations, we would predict that UD-CG 212 Cl would be unable to increase submaximal force because a minimal number of strong cross-bridges are available to activate neighboring functional units.

On the basis of our hypothesis that UD-CG 212 Cl enhances thin filament activation by adjacent strong cross-bridges, it was not surprising that the increased Hill *n* that we (Table II) and others (Kentish, 1986; Millar & Homsher, 1990; Nosek *et al.*, 1990) have observed with elevated P_i was not reversed by UD-CG 212 Cl. The effect of P_i on Hill *n* is postulated to occur because the reduced population of strong cross-bridges, which bind cooperatively to the thin filament, becomes limiting to myofilament activation (Millar & Homsher, 1990). Strong cross-bridges are not limiting at low P_i concentrations. Under this later condition, myofilament activation depends largely on the less cooperative process of Ca^{2+} binding to TnC (Millar & Homsher, 1990; Pan & Solaro, 1987).

The lack of effect on P_i-mediated changes in cooperativity by UD-CG 212 Cl (Figure 4, Table II) fits with our hypothesis that UD-CG 212 Cl does not directly act on the cross-bridge. Moreover, the lack of UD-CG 212 Cl effect on Hill *n* in the presence of P_i indicates it probably did not directly influence Ca^{2+}-mediated thin filament activation. Cooperativity if anything increased in response to UD-CG 212 Cl (Table II), which indicates that the drug may enhance the ability of strong cross-bridges to activate neighboring functional units (Figure 9).

Another way of changing the population of cross-bridges is to vary the ATP concentration (Brandt *et al.*, 1990; Bremel & Weber, 1972; Godt, 1974). Relatively low concentrations of ATP (e.g., 1 μM) result in a cross-bridge population high in rigor complexes. This situation is somewhat analogous to the previously discussed effects of low P_i concentration shown in Figure 9. These rigor complexes activate the thin filament independent of Ca^{2+} (Bremel & Weber, 1972; Metzger, 1992). As ATP is increased to 20 μM, the proportion of rigor linkages decreases, but adequate numbers of these linkages are present to activate thin filaments on neighboring functional units (Godt, 1974). In terms of the relationship depicted in Figure 9, this condition is similar to that found with moderately high P_i levels. When either P_i or ATP concentrations are slightly increased, cooperativity is primarily determined by the highly cooperative, rigor/strong cross-bridge activation of the thin filament. Further increases in ATP concentration to 5 mM virtually eliminate the presence of rigor cross-bridges (Figure 9; Brandt *et al.*, 1990; Bremel & Weber, 1972). Under this condition, Ca^{2+} binding to TnC limits activation of the thin filament.

The actions of UD-CG 212 Cl in the presence of low ATP concentrations also can be explained by the hypothetical scheme presented in Figure 9. UD-CG 212 Cl had minimal effects on force with 1 μM ATP (Figure 8A), a concentration in which most cross-bridges are bound as rigor linkages. The number of strong/rigor cross-bridges at this point is optimal, and we predict the drug would have no further effect (see Figure 9). It is also apparent from results obtained with 5 mM ATP without added P_i (Table I) that UD-CG 212 Cl is unable to enhance the activation of neighboring functional units when there is a large population of weakly-attached cross-bridges. However, when rigor/strong cross-bridges are limiting to activation of the thin filament (e.g., 20 μM ATP), UD-CG 212 Cl should increase force (Figure 8B, Table III). Thus, the increased tension produced by UD-CG 212 Cl in the presence of 20 μM ATP is consistent with our hypothesis that this drug acts to facilitate activation of the thin filament (Figure 9).

In conclusion, we think our results indicate that UD-CG 212 Cl represents a new pharmacological tool that aids myofilament activation with high affinity. Further elucidation of the myofibrillar structures affected by UD-CG 212 Cl are likely to provide important information about the mechanism of myofilament activation. Low doses of UD-CG 212 Cl may also be beneficial in the clinical treatment of myocardial ischemia. Inorganic phosphate increases progressively during myocardial ischemia and may reach 20 mM (Allen & Orchard, 1987; Kentish, 1991; Kusuoka *et al.*, 1986). Our results suggest UD-CG 212 Cl may have direct beneficial effects on the myofilament when severe myocardial ischemia develops. The direct myofilament effects of UD-CG 212 Cl may also

contribute to the clinical actions of the parent compound pimobendan (UD-CG 115 BS) (Przechera *et al.*, 1991).

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