Interaction Between Muscarinic Receptor Subtype Signal Transduction Pathways Mediating Bladder Contraction

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Running Title: MUSCARINIC RECEPTORS INTERACT IN BLADDER CONTRACTION
Abstract

M$_3$ muscarinic receptors mediate cholinergic induced contraction in most smooth muscles. However, in the denervated rat bladder, M$_2$ receptors participate in contraction, since M$_3$-selective antagonists (p-F-HHSiD and 4-DAMP) have low affinities. However, the affinity of the M$_2$-selective antagonist, methoctramine in the denervated bladder is consistent with M$_3$ receptor mediating contraction. It is possible that two pathways interact to mediate contraction; one mediated by the M$_2$ receptor and one by the M$_3$. To determine whether an interaction exists, the inhibitory potencies of combinations of methoctramine and p-F-HHSiD for reversing cholinergic contractions were measured. In normal bladders, all combinations gave additive effects. In denervated bladders, synergistic effects were seen with the 10:1 and 1:1 (methoctramine:p-F-HHSiD w/w) combinations. Following application of the sarcoplasmic reticulum ATPase inhibitor thapsigargin to normal tissue, the 10:1 and 1:1 ratios became synergistic, mimicking denervated tissue. Thus in normal bladders both M$_2$ and M$_3$ receptors can induce contraction. In the denervated bladder, the M$_2$ and the M$_3$ receptors interact in a facilitatory manner to mediate contraction.

Key words: Urinary Bladder, Synergy, Muscarinic Receptors, Denervation, Second Messengers

Abbreviations: p-F-HHSiD, para-fluoro-hexahydro-sila-diphenidol; AF-DX 116, [[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6$H$-pyrido[2,3$b$][1,4]benzodiazepine-6-one; 4-DAMP mustard, N-(2-chloroethyl)-4-piperidinyl dephenylacetate; PTX, pertussis toxin; IP$_3$, inositol triphosphate; PI, phosphatidyl inositol.
Pharmacologic data, based on the actions of subtype-selective antimuscarinic agents, can distinguish four subtypes of muscarinic acetylcholine receptors (M₁ - M₄). Molecular techniques have identified five muscarinic receptor subtypes (M₁-M₅) arising from five separate genes (1). Both M₂ and M₃ muscarinic receptor subtypes are found in most smooth muscles. The M₂ receptor preferentially couples to the inhibition of adenylyl cyclase through the Gᵢ family of proteins, while the M₃ receptor preferentially couples to IP₃ generation and calcium mobilization through the Gᵣ family of proteins (1). Pertussis toxin (PTX), which ADP ribosylates and therefore inactivates the Gᵢ family of proteins, has no apparent effect on contraction (2). Even though the M₂ muscarinic receptor density is greater than the M₃ receptor density in bladder and other smooth muscles, the affinity of subtype selective muscarinic receptor antagonist drugs indicates that contraction is mediated by the M₃ receptor in most smooth muscles under normal conditions (1,3).

A number of studies have shown that under certain conditions the M₂ receptor subtype can contribute to the contractile response. This includes selective alkylation of M₃ receptors in an environment of increased intracellular levels of cAMP in the rat urinary bladder (4,5), guinea pig ileum (6) and trachea (7) or following alkylation without having to raise intracellular cAMP levels in other tissues such as the guinea pig gallbladder (8) and colon (9). Other studies of smooth muscle contraction following experimentally induced pathologies, for example in a cat model of experimentally induced esophagitis (10), in the denervated rat bladder (11), and in a model of acute cholecystitis in the guinea pig gallbladder (8), also suggest that the M₂ receptor participates in mediation of contraction. In addition, in otherwise normal tissues, the M₂ receptor appears to mediate contraction following inhibition of the sarcoplasmic reticulum.
calcium ATPase, G\textsubscript{q} (10), phosphatidyl-inositol specific phospholipase C (PI-PLC), phophatidyl-choline specific phospholipase C (PC-PLC) or protein kinase C (12). Treatment with 4-DAMP mustard causes a greater decrease in muscarinic agonist affinity for stimulating contraction in guinea pig colon from animals exposed to PTX for 3 days than in non-PTX treated animals. However, 4-DAMP mustard is equally effective in decreasing agonist affinity for stimulating PI turnover in PTX treated animals compared to normal animals. This suggests that signal transduction mechanisms unrelated to PI turnover such as those activated by the M\textsubscript{2} subtype participates in the contractile response (2). Taken together these findings suggest that the M\textsubscript{2} and M\textsubscript{3} receptors, through different second messenger pathways, interact to result in smooth muscle contraction.

When two drugs with overtly similar actions are administered together, the combination may produce effects that are significantly greater (super-additive or synergistic) or less (sub-additive) than the simple additive effects calculated from the individual drug potencies. One explanation for a departure from additivity is an interaction between the drug targets involved in the response. To determine whether an interaction exists between the M\textsubscript{2} and M\textsubscript{3} receptor subtypes in normal urinary bladder tissue, the inhibitory potencies of combinations of methoctramine (M\textsubscript{2}-selective antagonist) and p-F-HHSiD (M\textsubscript{3}-selective antagonist) for reversing carbachol induced contractions by competitive antagonism (inhibition) was measured. Similar experiments using 3 day denervated rat bladder tissue were performed for comparison. Using the inhibitory potency of each drug alone, the theoretical additive potency of each combination of methoctramine and p-F-HHSiD (i.e. 10:1, 1:1, and 1:10 w:w of methoctramine to p-F-HHSiD) was determined. Comparison of the actual inhibitory potencies of these drug combinations with
those predicted from simple additivity allowed identification of certain antagonist combinations with inhibitory potencies that were significantly different from those predicted by simple additivity. These comparisons were made using both normal and denervated rat bladder. Because Sohn et. al. (10) have shown that treatment of lower esophageal sphincter smooth muscle cells with thapsigargin (sarcoplasmic reticulum calcium ATPase inhibitor) alters the contractile mechanism such that the M₂ muscarinic receptor is involved in mediation of contraction, these comparisons were also made in normal bladder exposed to thapsigargin. Revealing an interaction between the second messenger systems activated by the M₂ and M₃ receptor subtypes resulting in smooth muscle contraction may allow for the development of superior drugs for the treatment of overactive bladder or urinary incontinence and may help explain the clinical efficacy of the currently available antimuscarinic agents for the treatment of overactive bladder.
Materials: The following drugs or chemicals were obtained from the sources indicated: carbachol and thapsigargin (Sigma Chemical Company, St. Louis, Mo), methoctramine and p-F-HHSiD (Research Biochemicals International, Natick, MA).

Surgery: Rats (200-250 g female Sprague-Dawley rats from Ace Animals Inc., Boyertown, PA) were anesthetized with 2% isoflurane in oxygen and a midline incision was made in the lower abdomen. The pelvic plexus was exposed. For bilateral denervation, both the left and right major pelvic ganglion were electrocauterized with a Valleylab Inc. (Boulder, CO) hand stitching pencil attached to a Model SSE 2 solid state electrosurgery device (Valleylab Inc., Boulder, CO). For sham operated animals, the plexus was exposed but left intact. After surgery, because the denervated animals were unable to micturate, urine was expressed by manual pressure on the lower abdomen of the denervated and sham operated animals twice daily for three days.

Muscle Strips: Urinary bladders were removed from rats euthanized by decapitation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat; the epithelium was left in place. The bladder was divided in the mid-sagittal plane, then cut into longitudinal smooth muscle strips (approximately 4 mm x 10 mm). The muscle strips were then suspended with 1 g of tension in tissue baths containing 15 ml of modified Tyrode’s solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH2PO4, 1.8 mM CaCl2, 0.5 mM MgCl2, 23.8 mM NaHCO3, and 5.6 mM glucose) and equilibrated with 95/5% O2/CO2 at 37 oC. The muscle strips were tested for their ability to contract in response to electric field stimulation of 8 volts, 30 Hz, 1 ms duration. In order to determine cross sectional area,
conclusion of the experiment, the length and weight of each muscle strip between the suspension clips was measured. Cross sectional area was determined by weight/length which assumes a density of 1.0 for all muscle strips.

**Inhibition of Carbachol Response:** Following equilibration of the muscle strips to the bath solution for 60 minutes, 10 µM carbachol (approximately 10 times greater than the measured EC$_{50}$ value) was added to each bath. Once a stable baseline was reached (approximately 10 min), increasing concentrations of antagonist or combinations of antagonist were added every 5 minutes. For meaningful display of drug combinations, all combinations of antagonists are expressed as weight/weight ratios of methoctramine:p-F-HHSiD. For example an 11 µg/ml concentration of a 10:1 combination contains 10 µg/ml of methoctramine and 1 µg/ml of p-F-HHSiD resulting in a molar ratio of 5.2 moles of methoctramine per mole of p-F-HHSiD. Every experiment contained time control strips where Tyrodes solution was added in place of antagonist. The data is presented as the percent contraction at each antagonist concentration normalized to the contraction at 10 µM carbachol for 6-10 muscle strips per condition. During the course of the experiment, the tension in the time control strips gradually declined. As a result, the data was normalized by the percentage decline of the time control strips such that the contractile response of the control strips always equal 100 % contraction.

**Calculation of Potency for Inhibition Levels:** The potency of methoctramine, p-F-HHSiD and the combinations for the different inhibitory levels were calculated as described by Tallarida (13). Briefly, these potencies were determined using non-linear regression of the individual dose-effect curves. The predicted inhibitory potency of the combination is based on the inhibitory potencies of the individual antagonists, expressed as doses A and B, and the
combination doses a and b which together produce the same effect (50% inhibition of contraction) as dose A alone or B alone. An additive interaction relates these as, \( a/A + b/B = 1 \), while super-additivity and sub-additivity lead to the fraction sums being less than or greater than one, respectively. In other words, a sub-additive action is one that requires greater doses (a, b), whereas a super-additive action requires lower doses (a, b) to get the same degree of inhibition (50%). For example, in normal animals the IC\(_{50}\) for methoctramine was 4.57 µg/ml and the IC\(_{50}\) for p-F-HHSiD was 0.29 µg/ml. Following simple additivity, a combination of these two compounds that contains one-half the IC\(_{50}\) of methoctramine (2.28 µg/ml) and one-half the IC\(_{50}\) of p-F-HHSiD (0.15 µg/ml) would be expected to inhibit 50 % of contraction. If this combination produces significantly less than 50 % inhibition, then this combination is sub-additive. Conversely if this combination produces significantly greater than 50 % inhibition, this combination is synergistic. Significance between actual and predicted IC\(_{50}\) values was determined by the Student’s t-test with significance level \( p < 0.05 \).
Results

Denervated bladders weighed significantly more (p<0.05) than sham operated bladders (240 ± 15 mg vs. 96 ± 3 mg). However, muscle strips from denervated bladders contracted significantly less (p<0.05) to electric field stimulation (8 volts, 30 hz, 1 ms) than control strips (3.7± 0.4 mN/mm² and 18.9 ± 1.5 mN/mm², respectively). No differences in the maximal carbachol contraction were seen (sham, 33.9 ± 2.9 mN/mm²; denervated, 28.2 ± 2.2 mN/mm²). There were no significant differences in the contractile responses between normal bladders and bladders from sham-operated controls (data not shown), therefore, the data for normal and sham controls were pooled (Normal). As seen in figure 1, the addition of each of these muscarinic antagonists reversed the carbachol (10 µM) induced contraction in a concentration dependent manner. As shown in figure 2A, in normal rat bladder muscle strips, p-F-HHSiD (IC₅₀ 0.75 µM or 0.29 µg/ml) was just over 8 times more potent in reversing contraction than methoctramine (IC₅₀ 6.2 µM or 4.57 µg/ml). However, in muscle strips from denervated urinary bladders (Fig. 2B), p-F-HHSiD (IC₅₀ 4.3 µM or 1.67 µg/ml) was only 2 times more potent in reversing contraction than methoctramine (IC₅₀ 8.8 µM or 6.51 µg/ml). These results are consistent with our previous study showing that the affinity of p-F-HHSiD for inhibiting contraction decreased following denervation (11). Addition of 1 µM thapsigargin to normal tissue had no effect on the maximal carbachol induced contraction (43.7 ± 7.4 mN/mm² for thapsigargin treated versus 44.4 ± 4.8 mN/mm² for normal) or the potency of carbachol for inducing contraction (EC₅₀ = 2.25 ± 0.3 µM for thapsigargin treated versus 2.10 ± 0.3 µM for normal). However, thapsigargin treatment increased the IC₅₀ for methoctramine approximately 2 fold, from 4.57 µg/ml in normal to 9.7 µg/ml (13.3 µM). The IC₅₀ for p-F-HHSiD increased more than 5 fold, from 0.29 µg/ml in
normal to 1.58 µg/ml (4.1 µM).

Based on the individual inhibitory potencies of these antagonists, theoretical additive potencies for 50 % inhibition of contraction for the three fixed-ratio combinations, methoctramine:p-F-HHSiD (10:1, 1:1, and 1:10 w/w) were calculated. The character of the interaction is most easily seen by comparing the actual and calculated doses based on simple additivity (Fig. 2). If the actual combination dose is lower than the calculated additive dose, then there is super-additivity (synergism); if it is higher, then there is sub-additivity. In bladder strips from normal animals, all combinations were additive. The results from denervated animals were quite different. The 10:1 and 1:1 combinations were synergistic while the 1:10 combination was additive. Likewise, in bladder strips from normal animals treated with 1 µM thapsigargin, both the 10:1 and the 1:1 combinations were super-additive, while the 1:10 combination was additive, thus appearing more similar to the denervated preparation than the normal preparation.
Discussion

Additivity of 2 antagonists implies independence, i.e. one agent may be substituted for the other in an amount that is calculated from their relative inhibitory potencies. A finding of sub-additivity is consistent with a mechanism in which the two antagonists inhibit two pathways that are activated by the agonist and that attenuate each other. Alternatively, a finding of sub-additivity could also be seen with two compounds that compete for a common receptor. Conversely, a finding of super-additivity is consistent with the two antagonists inhibiting two pathways that inhibit each other. In the normal rat bladder, the finding that all combinations of the two antagonists displayed simple additivity suggests that the contractile pathways activated by the M₂ and M₃ receptor subtypes appear to be functioning independently and that the two antagonists are sufficiently selective such that competition for the same receptor subtype (sub-additivity) is not seen under these conditions.

Evidence for the existence of an interaction between the signal transduction pathways activated by the M₂ and the M₃ receptor subtypes is seen with normal rat bladder strips in the presence of thapsigargin. Thapsigargin treatment had no effect on the maximal carbachol induced contraction or the potency of carbachol for inducing contraction. However, thapsigargin treatment altered the mechanism of contraction such that the 10:1 and the 1:1 combinations of methoctramine:p-F-HHSiD which were additive in normal (fig. 2A) became super-additive after treatment (fig. 2C), a change similar to the effects of denervation. We performed immunoprecipitation assays to confirm that thapsigargin exposure for 30 minutes did not change the density of muscarinic receptor subtypes unlike denervation, where the density of M₂ receptors increased and the density of M₃ receptors decreased (12,14). Thus, at least for this
pharmacological manipulation (in-vitro thapsigargin exposure), the change in the mechanism of smooth muscle contraction mediated by M2 and M3 muscarinic receptors is not related to changes in receptor densities. The results of this series of experiments support the hypothesis that two interacting contractile mechanisms exist in normal tissue, one mediated by the M2 receptor subtype and the other mediated by the M3 receptor subtype, which may interact through calcium mobilization. While the interaction of the contractile pathways activated by the M2 and the M3 receptor subtypes is apparently masked in normal tissue, treatment with thapsigargin reveals the interaction. These results provide functional evidence that both the M2 and the M3 receptor subtypes are found on smooth muscle cells and mediate contraction.

M2 receptors are traditionally thought to preferentially couple to PTX sensitive G proteins such as the G\textsubscript{i} subfamily, resulting in inhibition of adenylyl cyclase. An M2 mediated contractile response in bladder muscle can be demonstrated after the majority of M3 receptors are inactivated in an environment of increased intracellular cAMP such as during stimulation with a \(\beta\) adrenergic agonist (4,5). This pathway has been proposed to mediate contraction indirectly, merely by blocking \(\beta\) adrenergic agonist induced relaxation via increased cAMP (3). However, M2 receptors acting through G\textsubscript{i} may also stimulate bladder contraction directly via PKC activation (15). As found in the cat lower esophageal sphincter, a low degree of muscarinic stimulation, and consequently, a low degree of calcium mobilization, results in activation of PKC, whereas PKC activation is inhibited at higher intracellular calcium concentrations (16). Thus, in the face of normal calcium mobilization mediated by the M3 receptor subtype, the signal transduction pathway mediated by the M2 subtype may be inhibited. This may explain the finding that in the presence of thapsigargin, which interferes with calcium signaling, the signal
transduction pathways activated by the M_2 and the M_3 receptor subtype interact in a facilitatory manner to induce contraction.

The denervated preparation gave markedly different results than normal tissue. Here the combinations with the highest proportion of the M_2-selective antagonist methoctramine (fig. 2B) showed a synergistic inhibitory effect that reverted to simple additivity when its proportion in the mixture was reduced to one-tenth that of the M_3-selective antagonist p-F-HHSiD. These results in the denervated preparation support an interaction between subtypes in the model reported by Sawyer et. al. (2) which predicts that the M_2 and M_3 receptor subtypes interact in a facilitatory manner to mediate contraction of the guinea pig colon. However, our results in the normal rat bladder do not support a facilitatory interaction between muscarinic receptor subtypes for inducing contraction. Only after either denervation or thapsigargin treatment does this interaction become facilitatory. It is possible that the interaction between subtypes is different in the guinea pig colon, or that after three days of in-vivo PTX treatment, the interaction between subtypes becomes altered. Denervation results in a two-fold increase in the density of M_2 receptors while the density of M_3 receptors decreases about 50% (12,14). It is possible that the alteration in the mechanism of contraction in the denervated preparation is related to changes in receptor density.

Does the M_3 pathway inhibit the M_2 pathway; does the M_2 pathway inhibit the M_3 pathway; or do both pathways inhibit each other? Synergistic analysis as performed in this study does not distinguish between these scenarios. However, some insight may be derived from the experiments with normal rat bladder in the presence of thapsigargin. In the presence of thapsigargin, the interaction of the subtypes not only becomes super-additive, but subtype
selective antagonist affinities suggests that the M$_2$ receptor subtype is involved in mediation of contraction (12). Our interpretation is that thapsigargin inhibits some component of the M$_3$ contractile pathway, consequently the M$_2$ pathway is not inhibited and thus mediates contraction. If activation of the M$_2$ pathway inhibited the M$_3$ pathway to a significant extent, one would predict that the M$_2$ pathway would mediate contraction following selective alkylation of M$_3$ receptor subtypes. However, when the majority of the M$_3$ receptor subtypes in the rat bladder are inactivated by alkylation, and intracellular cAMP is not elevated, antagonist affinities remain consistent with M$_3$ receptor mediated contraction, suggesting that relatively low levels of M$_3$ receptor activation are sufficient to inhibit the M$_2$ mediated response. Consequently, the data presented here for normal tissue without thapsigargin, suggests that no interaction between subtypes or their signal transduction systems exits. When analyzed in context with the results of the denervated and thapsigargin treated tissue, we speculate, that in normal bladder smooth muscle, the M$_3$ contractile pathway inhibits the M$_2$ contractile pathway such that only the M$_3$ pathway is evoked.

A possible explanation for why the antagonists in combination did not show sub-additive effects in normal tissue is because these experiments were performed with pre-stimulation of muscarinic receptors which raised intracellular calcium and thus inactivated the M$_2$ pathway before the addition of the antagonists. Since these antagonists are only about 10-fold selective, it may not be possible to lower intracellular calcium enough to allow reactivation of the M$_2$ pathway without also inhibiting it at the receptor level. Our previous receptor inactivation studies with 4-DAMP-mustard alkylation in normal tissue demonstrated that very low levels of the M$_3$ receptor subtype are sufficient to mediate contraction (4). This suggests that virtually all
M₃ receptor activation must be blocked in normal tissue, or a component of the signal transduction pathway mediated by the M₃ receptor subtype must be blocked with an inhibitor such as thapsigargin, in order to reveal the contractile pathway activated by the M₂ receptor subtype.

It is possible that inhibition of the M₂ pathway by the M₃ pathway in normal bladders is the result of one subtype inducing the release of a relaxant factor and that following denervation or thapsigargin treatment, the release of this factor is blocked and once blocked, the M₂ and M₃ receptor subtypes cooperate in a positive manner to result in contraction. However, if one subtype mediates the release of a relaxant factor, while the other subtype induces smooth muscle contraction, then as the antagonist inhibits the release of this inhibitory factor an increase in the magnitude of contraction would be expected. Our data (collected with the epithelium in place) are not consistent with this scenario, since no increase in the magnitude of contraction with any doses of antagonist or antagonist combinations occurred either in normal, thapsigargin treated or denervated bladders. In the rat urinary bladder, no urothelial relaxant factor has been identified, however this has been shown in the pig bladder (17). However, Fovaeus et. al. (18) describe a relaxant factor derived from rat bladder which is not of epithelial origin.

The above results support the existence of two independent contractile pathways in normal bladder smooth muscle and the interaction between these pathways changes significantly following denervation or inhibition of calcium mobilization. In the denervated rat bladder, the finding of a super-additive inhibition with the combinations suggests that the M₂ and M₃ receptor (or the second messenger systems activated) interact in a facilitatory manner to mediate contraction. Therefore, following denervation, the contractile pathways activated in response to
muscarinic stimulation change from inhibitory to facilitatory. This finding, if confirmed in human bladder, has important implications regarding the design of antimuscarinic agents for urologic use and may help explain the clinical efficacy of the M₂ - M₃ selective antimuscarinic drug tolterodine (19), recently approved by the FDA for treatment of an overactive bladder. This is based on the recently proposed concept that bladder overactivity results from varying degrees of functional or actual motoneuron denervation of the detrusor (20). Our application of synergistic analysis to antagonist inhibition, usually used in agonist combination analysis, has provided strong quantitative evidence for the joint roles of both M₂ and M₃ muscarinic receptor subtypes in smooth muscle contraction.
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References:


16. **Sohn UD, Chiu TT, Bitar KN, Hillemeier C, Behar J and Biancani P.** Calcium requirements for acetylcholine-induced contraction of cat esophageal circular muscle


Figure Legends:

Fig. 1. Inhibition of carbachol (10 µM) induced contraction of normal, denervated and thapsigargin treated rat urinary bladder muscle strips in the presence of methoctramine, p-F-HHSiD, and combinations of methoctramine and p-F-HHSiD. Data is presented as mean ± S.E.M. of 6-10 muscle strips from at least 4 different animals per condition. A, Normal, methoctramine - squares, Hill slope - 1.06 ± 0.09; p-F-HHSiD - circles, Hill slope - 0.76 ± 0.08 *; 10:1 - triangles, Hill slope - 1.09 ± 0.03; 1:1 - upside down triangles, Hill slope 0.93 ± 0.09; 1:10 - diamonds, Hill slope - 0.76 ± 0.06 *. B, denervated, methoctramine - squares, Hill slope - 0.65 ± 0.08 *; p-F-HHSiD - circles, Hill slope - 0.64 ± 0.05 *; 10:1 - triangles, Hill slope - 0.96 ± 0.07; 1:1 - upside down triangles, Hill slope 1.00 ± 0.04; 1:10 - diamonds, Hill slope - 0.88 ± 0.04 *. C, thapsigargin treated, methoctramine - squares, Hill slope - 0.82 ± 0.09; p-F-HHSiD - circles, Hill slope - 0.70 ± 0.07 *; 10:1 - triangles, Hill slope - 0.79 ± 0.03; 1:1 - upside down triangles, Hill slope 0.96 ± 0.07; 1:10 - triangles, Hill slope - 0.81 ± 0.06 *. * denotes Hill slope is significantly different from 1.0 (p < 0.05).

Fig. 2. Average IC$_{50}$ for inhibition of carbachol (10 µM) induced contraction of normal (A) denervated (B) and thapsigargin treated (C) rat urinary bladder muscle strips in the presence of methoctramine, p-F-HHSiD and combinations of methoctramine and p-F-HHSiD along with the predicted additive inhibitory potencies of the combinations (weight:weight ratios of methoctramine:p-F-HHSiD). Data was derived from figure 1 and displays the mean ± SEM for doses that cause 50% inhibition (IC$_{50}$) of contraction induced by 10 µM carbachol. * denotes significant difference (p < 0.05) between the predicted inhibitory potency based on simple additivity and the actual inhibitory potency of the combination (Student’s t-test).
Figure 1

A-Normal

B-Denervated

C-Normal, Thapsigargin Treated

% 10 μM Carb Contraction

[antagonist] (μg/ml)

Meth
p-F-HHSiD
Combinations
△ 10:1
▼ 1:1
● 1:10
Figure 2