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Gastric body cholinergic contractile signal transduction in M₂ and M₃ receptor knockout mice

Michael R. Ruggieri Sr. and Alan S. Braverman

Departments of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, Pennsylvania

Abstract

Although most smooth muscles express a greater density of M₂ than M₃ muscarinic receptors, based on the potency of subtype selective muscarinic receptor antagonists, the M₃ subtype predominantly mediates contraction. The effect of inhibitors of putative contractile signal transduction pathway enzymes on carbachol induced contractions was determined in wild type mice (WT) and mice lacking either the M₂ (M₂KO) or the M₃ (M₃KO) receptor subtype. Contractile responses to KCl, then increasing carbachol concentrations in the presence and absence of enzyme inhibitors was determined. The KCl induced contraction was not different between strains. The carbachol response was unaffected in the M₂KO strain but decreased 42% in M₃KO mice (p<0.01). Darifenacin potency was high in both WT and M₂KO strains, indicating M₃ mediated contractions, and low in the M₃KO strain, suggesting M₂ mediated contractions. The phosphatidyl inositol specific phospholipase C (Pi-PLC) inhibitor ET-18-OCH₃ had no effect. Inhibition of phosphatidyl choline specific phospholipase C (PC-PLC) and sphingomyelin synthase with D609 decreased maximal contraction in all strains. M₃ mediated contractions in the M₂KO strain were decreased 54% by the protein kinase C (PKC) inhibitor chelerythrine. M₂ mediated contractions in the M₃KO and WT strains were decreased by the Rho kinase (ROCK) inhibitor Y27632 as well as the ROCK, PKA and PKG inhibitor H89. The M₃ subtype activates PKC and either PC-PLC or sphingomyelin synthase, while the M₂ subtype activates ROCK and either PC-PLC or sphingomyelin synthase. These studies suggest that multiple parallel pathways mediate cholinergic contractions in stomach body smooth muscle.

Key terms

muscarinic receptors; signal transduction; smooth muscle; phospholipases; Rho kinase; sphingomyelin synthase

INTRODUCTION

Gastric emptying is a carefully regulated process involving the fundus, body, and antrum components of the stomach. Gastric emptying is mediated through cholinergic pathways since atropine, a muscarinic antagonist, slows murine gastric emptying (1).

Corresponding Author: Michael R. Ruggieri, Sr., Ph.D., Temple University School of Medicine, 3400 North Broad Street, Philadelphia, Pa 19140. Telephone: (215) 707-4567, Fax: (215) 707-4565, rugg@temple.edu.

DECLARATION OF INTEREST STATEMENT:

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There are known to be five subtypes of muscarinic receptors, M₁, M₂, M₃, M₄, and M₅ (2, 3). Cholinergic contractions of gastrointestinal (GI) smooth muscle are primarily receptor subtype. However, the majority of muscarinic receptors mediated through the M₃ receptor subtype (4). In the urinary bladder, in the GI tract have been found to be the M₂ although cholinergic contractions are predominately M₃ receptor mediated, the M₂ subtype contributes to muscarinic mediated contraction in rats (5, 6) mice (7, 8) and humans (9). Whether there is an M₂ mediated contractile component in the normal mouse stomach and whether there is an interaction between M₂- and M₃-mediated contractile signal transduction pathways is not known.

The aims of this study were twofold: first, to determine the subtypes of muscarinic receptors mediating cholinergic contractions of the stomach using M₂ and M₃ receptor knockout (KO) mice; second, to explore the contractile signal transduction cascades activated by M₂ and M₃ receptors.

METHODS

Materials

The following drugs or chemicals were obtained from the Sigma Chemical Company (St. Louis, Mo.): carbachol, (R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632), N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), 1,2-Dimethoxy-N-methyl(1,3)benzodioxolo(5,6-c) phenanthridinium chloride (chelerythrine), 1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine (ET), O-Tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate potassium salt (D-609) Darifenacin (DAR) was a generous gift from Pfizer Limited (Sandwich, Kent). The target enzymes, K_i for the enzyme inhibitors and the concentration used are listed in table 1. M₂KO, M₃KO and their respective WT strains of mice were a kind gift from Dr. Jurgen Wess, director of the Laboratory of Bioorganic Chemistry, National Institutes of Diabetes, Digestive and Kidney Diseases.

Muscle Strips

Stomachs were removed from mice euthanized by CO₂ asphyxiation. Both the fundus and the antrum were removed, the stomach body was opened along the long axis and muscle strips were cut aligned with the circular muscle fibers (approximately 2 mm × 5 mm). The muscle strips were then suspended with 0.5 g of tension in tissue baths containing 10 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 °C.

Carbachol Concentration Response

Following equilibration to the bath solution for 30 minutes, the contractile response induced by isotonic Tyrode's solution containing 120 mM potassium was recorded. Sixteen separate muscle baths were run simultaneously. The strips were ranked based on their contractile response to KCl to sort into the different drug treatment groups such that the mean contractile response to KCl was similar for each group of strips. The strips were incubated for 30 minutes in the presence or absence of an enzyme inhibitor and in the presence or absence of either 10 or 30 nM darifenacin. Higher concentrations of darifenacin appeared non-competitive and lower concentrations did not produce a significant shift in the concentration response curve, thus, these two concentrations of darifenacin were used to construct Schild plots to determine potency. Concentration response curves (CRC) were derived from the peak tension developed following cumulative addition of carbachol (10 nM

to 300 μ M final bath concentration) and normalized to the initial 120 mM KCl response. Strips were then washed 3 times and after a 20 minute re-equilibration period, the contractile response induced by 120 mM KCl was again recorded. Only one concentration of enzyme inhibitor or darifenacin was used for each muscle strip and thus each strip was only exposed to a single agonist CRC. Concentration ratios were determined based on the average responses of antagonist free strips. Because some of the enzyme inhibitors decreased the maximal contraction to less than 50 % of the KCl response, darifenacin potency was calculated based on both EC₂₅ values derived from normalization to the KCl response and EC₅₀ values derived from normalization to the maximal carbachol response for each muscle strip. The EC values were determined for each strip using a sigmoidal curve fit of the data (Origin, Originlab Corp. Northampton, MA).

Because there were statistically significant differences in the variance between groups, statistical analysis was performed by non-parametric Mann-Whitney U test for pair wise comparisons (GB-STAT, Dynamic Microsystems, Silver Spring, MD). Because there were no statistically significant difference between the M₂ wild type and the M₃ wild type strains, the data for these strains was pooled for comparisons to the knock out strains.

RESULTS

In order to determine if the muscle strips from each strain of animals is able to generate the same force, the contraction generated by 120 mM KCl was determined for each muscle strip. There were no statistically significant differences between the 120 mM KCl induced tension between the WT, M₂KO and the M₃KO strains. In time control strips, responses to 120 KCl at the conclusions of the carbachol CRC were 69 $\pm$ 6% of the initial KCl response and there were no statistically significant differences between strains. None of the enzyme inhibitors or darifenacin had any effect on the final KCl responses. The maximal response elicited by carbachol was significantly lower ($p < 0.01$) in the M₃KO stomach strips (34.5 % of KCl response, $n=21$) compared to stomach strips from either the WT (82.1% of KCl response, $n=25$) or the M₂KO (78.4 % of the KCl response, $n=13$) strains. There was no difference in the maximal carbachol stimulated contraction between WT and M₂KO animals (figures 1A and 3). Based on the EC₂₅ of the KCl response there was no difference in the potency of carbachol between any of the strains, however based on the EC₅₀ of the maximal carbachol response, carbachol potency was significantly ($p < 0.05$) lower in the M₂KO strain compared to the M₃KO strain (figure 1B).

The potency of darifenacin for inhibition of carbachol induced contractions in wild type stomach strips was calculated by constructing Schild plots for the data normalized to the percent of the contraction to 120 mM KCl and to the percent of the maximal contraction for each strip. Table 2 shows potency values calculated using EC₅₀ values determined using percent of the maximal carbachol induced contraction for each strip. In both cases, the estimated pK_b was high, consistent with the M₃ receptor subtype mediating contraction. Darifenacin potency in stomach body muscle from M₂KO animals was also high, consistent with M₃ mediated contractions (table 2). The potency of darifenacin was much lower in M₃KO animals because neither 10 nM nor 30 nM darifenacin caused a significant shift in the carbachol concentration response curve (data not shown).

In stomach muscle from wild type and M₃KO animals, inhibition of ROCK with Y-27632, inhibition of PKA, PKG, and ROCK with H89, or inhibition of PC-PLC and sphingomyelin synthase with D609 decreased the maximal carbachol response. In the M₂KO strain, inhibition of PKC with chelerythrine and inhibition of PC-PLC and sphingomyelin synthase with D609 decreased the maximal response.

For comparison of carbachol potency, the pEC_{50} (negative log of the carbachol concentration which yields half the maximal response) for muscle strips exposed to vehicle was compared to the muscle strips exposed to an enzyme inhibitor (table 2). In WT stomach muscle strips, inhibition of ROCK with Y-27632 statistically significantly decreased carbachol potency compared to vehicle. In M_2 KO mice, inhibition of ROCK with Y-27632, inhibition of PKA, PKG and ROCK with H89 and inhibition of PC-PLC with D609 statistically significantly decreased carbachol potency compared to vehicle. In the M_3 KO bladder strips, inhibition of ROCK with Y-27632 and inhibition of PC-PLC with D609 statistically significantly decreased carbachol potency.

Darifenacin potency following enzyme inhibition was calculated using the EC_{50} data as described above. For these studies only a single concentration of darifenacin was used (10 nM), thus the estimated pK_b for darifenacin was calculated using the formula $pK_b = -(\log [\text{darifenacin concentration}] - \log (\text{concentration ratio} - 1))$. None of the enzyme inhibitors caused a significant change in darifenacin potency for inhibition of carbachol induced stomach body muscle contraction in either WT or M_2 KO stomach strips. Darifenacin potency in M_3 KO stomach strips was low. Because 10 nM darifenacin induced no dextral shift in the carbachol concentration curves in M_3 KO bladder strips, darifenacin potency must be less than 8 but the exact potency could not be accurately calculated.

DISCUSSION

Our studies demonstrate that cholinergic contractions of the gastric body are primarily mediated by the M_3 receptor subtype with a smaller contribution by the M_2 subtype. The M_3 subtype activates either PC-PLC or sphingomyelin synthase and PKC pathways to mediate gastric smooth muscle contraction. The M_2 subtype activates either PC-PLC or sphingomyelin synthase and Rho kinase pathways to mediate gastric smooth muscle contraction. Similar to findings in urinary bladder smooth muscle from mouse (10), rat (11–13) and human (14); PI-PLC activation is not required for muscarinic receptor mediated contraction of the mouse stomach body. Thus, several parallel pathways mediate cholinergic contractions in gastric smooth muscle. At the receptor level, the cholinergic contractions are primarily M_3 mediated but also have an M_2 mediated component. At the signal transduction level, the contractions involve either PC-PLC or sphingomyelin synthase and PKC as well as Rho Kinase.

Absence of either the M_2 or the M_3 receptor has no effect on the ability of mouse stomach body muscle to contract in response to depolarization by an isotonic buffer containing 120 mM KCl. This suggests that any differences in the maximal response to the muscarinic receptor agonist carbachol are the result of differences in the expression of the muscarinic receptor subtypes in the knock out animals. In response to carbachol, stomach muscle from M_3 KO animals produces a contraction approximately 40% of the magnitude of the contraction from either WT or M_2 KO animals and this finding is in general agreement with previously published data (7, 8). Although the M_2 receptor is capable of eliciting contraction, our data suggests that the M_3 receptor subtype is required to mediate a maximal contraction. In contrast, in the bladder, the M_2 receptor subtype is only capable of mediating a minimal contraction, approximately 6% of the contraction of WT bladders (10, 15). As will be discussed below, the difference in the ability of the M_2 receptor to mediate contraction in the stomach and the bladder may be due to differences in the signaling cascades induced by M_2 receptor activation in these two different smooth muscles.

There is no difference in carbachol potency for inducing contraction between WT and either knock out strain, even though the carbachol maximal contraction is significantly reduced in M_3 KO stomach smooth muscle. Similar to previously published data (16) we found that the

maximal carbachol contraction was not attenuated, but carbachol potency was significantly lower in M₂KO as compared to M₃KO stomach strips. This finding is different than we found in the bladder from knockout animals, where deletion of the M₂ receptor subtype slightly but significantly attenuated the maximal carbachol induced contraction with no effect on potency (10). These differences indicate that muscarinic receptor mediated smooth muscle signal transduction mechanisms mediating contraction is organ dependent within a species such as the mouse.

Based on the effect of the enzyme inhibitors used in this study on the maximal carbachol response, PKC and either PC-PLC or sphingomyelin synthase appear essential for transducing the M₃ receptor mediated contractile signal transduction cascade. PI-PLC, PKA, PKG and ROCK are either not utilized in transducing the M₃ mediated contractile signal or are not essential for generation of a maximal response because of possible alternate parallel contractile pathways. The results from M₃KO animals are quite different. Inhibition of ROCK with either Y-27632 or H89 attenuated the maximal response while inhibition of PKC had no effect. This data suggests that ROCK is essential for the M₂ receptor mediated signal transduction cascade while PKC is either not essential or that alternate parallel contractile pathways activated by the M₂ receptor subtype substitute for PKC.

Based on the potency of darifenacin for inhibition of contraction in WT stomach smooth muscle, the M₃ receptor subtype mediates carbachol induced contraction. Inhibition of ROCK, with either Y-27632 or H89 or inhibition of either PC-PLC or sphingomyelin synthase with D609 attenuates the maximal contraction. Inhibition of PKC reduced the maximal response, but not to a statistically significant extent (n= 25 strips for vehicle and 13 for chelerythrine, p = 0.094). Thus, of the enzymes examined in this study, only ROCK and PC-PLC are essential in mediating contraction in WT. If the assumption is made that only the M₃ receptor subtype mediates contraction of stomach smooth muscle from WT animals, the data presented here from the knock out animals is contradictory. The results from the M₃KO animals matches the data from the WT animals (both ROCK and PC-PLC are essential) and is different from M₂KO animals (PKC and PC-PLC or sphingomyelin synthase are essential). This apparent contradiction is likely the result of cross talk between the signal transduction mechanisms when both M₂ and M₃ receptors can be activated in WT animals. The purportedly PC-PLC inhibitor D609 has been shown to inhibit sphingomyelin synthase at 187 μ M compared to 100 μ M used in our study (17). Sphingomyelin synthase catalyzes the breakdown of phosphatidylcholine to diacylglycerol while converting ceramide to sphingomyelin. Sphingomyelin breakdown products including sphingosylphosphorylcholine and sphingosine-1 phosphate can activate additional pathways including rho kinase (18, 19). Activation of muscarinic receptors has been shown to activate this sphingomyelin mediated pathway (20, 21). Thus the possibility exists that sphingomyelin synthase and not PC-PLC is essential for generation of a maximal carbachol stimulated contraction.

In the bladder, ROCK and PC-PLC are essential in order to obtain a maximal contraction in WT, M₂KO and M₃KO animals (10). In the stomach, both ROCK and PC-PLC are essential for M₃ mediated contractions when the M₂ receptor is present (WT). However, in the stomach, PKC and either PC-PLC or sphingomyelin synthase appear essential for M₃ mediated contractions, when no M₂ receptor is present, even though inhibition of PKC has no effect on the maximal contraction when the M₂ receptor is present. The stomach contracts significantly greater than the bladder from M₃KO animals even though the M₂ receptor utilizes the same signal transduction enzymes in these tissues. One possible explanation is that the M₂ receptor couples more efficiently to these enzymes in the stomach than the bladder. Confirmation of this explanation awaits further study. The above results suggest that different signaling cascades in different smooth muscle organs in the same

species are utilized by the same muscarinic receptor subtypes. These differences are likely responsible for the different maximal carbachol induced contraction in the stomach and bladder from M₃KO animals.

CONCLUSIONS

In conclusion, our results suggest differences in muscarinic receptor activated signal transduction mechanisms between different organs within the same species. Comparison of the data presented here with studies involving contractile signal transduction mechanisms from the same organ from different species suggests that there is also heterogeneity of contractile mechanisms between species in the same organ.

ABBREVIATIONS

CRC	concentration-response curve
D609	O-Tricyclo[5.2.1.0 ^{2,6}]dec-9-yl dithiocarbonate potassium salt
ET	1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine
H89	N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
M₂KO	M ₂ receptor knockout mice
M₃KO	M ₃ receptor knockout mice
PC-PLC	phosphatidyl choline specific phospholipase C
PI-PLC	phosphatidyl inositol specific phospholipase C
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
ROCK	Rho kinase
WT	wild type mice
Y27632	(R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride

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Figure 1.

Cumulative carbachol concentration effect curves normalized to each individual muscle strip's contractile response to 120 mM KCl (A) or each strip's maximal response to carbachol (B). WT, n=19; M₂KO, n=12; M₃KO, n=19.

Figure 2.

Effect of enzyme inhibitors on carbachol induced contractions from WT (A), M₂KO (B) and M₃KO mouse stomachs. WT, vehicle n=19, Y-27632 n=11, H89 n=10, chelerythrine n=12, ET n=16, D609 n=8; M₂KO, vehicle n=12, Y-27632 n=7, H89 n=7, chelerythrine n=8, ET n=6, D609 n=6; M₃KO, vehicle n=19, Y-27632 n=10, H89 n=6, chelerythrine n=6, ET n=10, D609 n=8.

Figure 3.
Effect of enzyme inhibitors on the maximal carbachol induced contraction as shown in figure 2 from WT (A), M₂KO (B) and M₃KO mouse stomachs. * denotes $p < 0.05$, ** denotes $p < 0.01$.

Table 1K_i for inhibitors at various enzymes (μM)

Inhibitor	PKA	PKC	PKG	ROCK	PL-PLC	PC-PLC	μM
Y-27632	25	26		0.1			10
H89	0.05		0.5	0.27			10
Chelerythrine		0.66					10
ET					5		100
D609						94	100

Table 2

Summary of the effect of enzyme inhibitors on carbachol and darifenacin potency.

Enzyme Inhibitor	Wild Type			M ₂ KO			M ₃ KO		
	Carbachol pEC ₅₀ (μM)	Darifenacin pK _b	Carbachol Max (%KCl)	Carbachol pEC ₅₀ (μM)	Darifenacin pK _b	Carbachol Max (%KCl)	Carbachol pEC ₅₀ (μM)	Darifenacin pK _b	Carbachol Max (%KCl)
Vehicle	6.1±0.12(25)	8.5±0.2(7)	85±8	5.7±0.17(13) #	8.6±0.2(6)	78±12	6.5±0.32(15)#	-	34±5##
Y-27632	5.8±0.13(12)*	8.7±0.25(8)	37±7***	5.1±0.06(6)*	-	104±22	5.4±0.26(6)*	-	12±3***
H89	6.4±0.15(12)	8.9±0.35(4)	46±8**	5.1±0.08(7)*	-	70±16	6.6±0.19(4)	-	13±2*
Chelerythrine	5.9±0.18(13)	8.5±0.3(5)	58±9	5.4±0.18(7)	-	45±12*	6.0±0.54(4)	-	31±10
ET	5.9±0.14(12)	8.3±0.21(0)	85±13	5.5±0.12(5)	8.4±0.24(3)	128±19	6.4±0.24(4)	-	19±6
D609	5.7±0.14(13)	8.3±0.01(2)	32±7***	4.5±0.18(5)**	-	24±9*	5.3±0.27(6)**	-	13±3*