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Title Page

M_2 and M_3 Muscarinic Receptor Activation of Urinary Bladder Contractile Signal Transduction. I. Normal Rat Bladder

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Running title: Normal Bladder M_2 and M_3 Contractile Signal Transduction

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Abbreviations: cAMP, cyclic adenosine monophosphate; CPI-17, Protein kinase C-potentiated phosphatase inhibitor of 17 kDa; CRC, concentration response curve; DAG, diacylglycerol; IP_3, inositol 3,4,5-triphosphate; MYPT-1, regulatory myosin phosphatase targeting sub-unit 1; MLC, myosin light chain; PC-PLC, phosphatidyl choline specific phospholipase C; PKC, protein kinase C; PKG, protein kinase G; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI-PLC, phosphoinositide specific phospholipase C; PKA, protein kinase A; PKG, protein kinase G; PLA_2, Phospholipase A_2; PLD, Phospholipase D; RGS, regulator of G-protein signaling, ROCK, rho kinase; SR, sarcoplasmic reticulum.

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ABSTRACT:

The muscarinic receptor subtype activated signal transduction mechanisms mediating rat urinary bladder contraction are incompletely understood. M_3 mediate normal rat bladder contractions, however, the M_2 receptor subtype has a more dominant role in contractions of the hypertrophied bladder. Normal bladder muscle strips were exposed to inhibitors of enzymes thought to be involved in signal transduction in-vitro followed by a single cumulative concentration response curve to the muscarinic receptor agonist carbachol. The outcome measures were the maximal contraction, the potency of carbachol, and the affinity of the M_3 selective antimuscarinic agent darifenacin for inhibition of contraction. Inhibition of PI-PLC with ET-18-OCH_3 reduces carbachol potency and reduces darifenacin affinity, while inhibition of PC-PLC with D609 attenuates the carbachol maximal contraction. Inhibition of rho kinase with Y-27632 reduces carbachol potency and increases darifenacin affinity. Inhibition of rho kinase, PKA and PKG with HA-1077 reduces the carbachol maximal contraction, carbachol potency and darifenacin affinity. Inhibition of PKC with chelerythrine increases darifenacin affinity while inhibition of rho kinase, PKA, PKG, and PKC with H7 reduces the carbachol maximum, carbachol potency while increasing darifenacin affinity. Inhibition of rho kinase, PKA and PKG with H89 reduces carbachol maximum and carbachol potency. Both the M_2 and the M_3 receptor subtype are involved in normal rat bladder contractions. The M_3 subtype appears to mediate contraction by activation of PI-PLC, PC-PLC, and PKA, while the M_2 signal transduction cascade may include activation of rho kinase, PKC, and an additional contractile signal transduction mechanism independent of rho kinase or PKC.

INTRODUCTION

Pharmacologic data, based on the actions of subtype-selective antimuscarinic agents, can distinguish four subtypes of muscarinic acetylcholine receptors (M_1 - M_4). Molecular techniques have identified five muscarinic receptor subtypes (M_1-M_5) arising from five separate genes (Caulfield, 1993). Both M_2 and M_3 muscarinic receptor subtypes are found in most smooth muscles. The M_2 receptor preferentially couples to the inhibition of adenylyl cyclase through the Gi family of proteins, while the M_3 receptor preferentially couples to IP_3 generation and calcium mobilization through the Gq family of proteins (Caulfield, 1993). Pertussis toxin (PTX), which ADP ribosylates and therefore inactivates the G_i family of proteins, has no apparent effect on contraction (Sawyer and Ehlert, 1999). Even though the M_2 muscarinic receptor density is greater than the M_3 receptor density in bladder and other smooth muscles, the affinity of subtype selective muscarinic receptor antagonist drugs indicates that contraction is mediated by the M_3 receptor in most smooth muscles under normal conditions (Caulfield, 1993; Sawyer and Ehlert, 1999). While not directly involved in mediating smooth muscle contraction, we and others have shown that prejunctional M_1 receptors mediate augmentation of neuronal acetylcholine release and M_2 receptors inhibit acetylcholine release (Somogyi and de Groat, 1992; Braverman et al., 1998a).

Subsequent steps in the contractile signal transduction pathway are not known for certain in bladder smooth muscle but one possibility involves $G_{q/11}$ activation of phosphatidylinositol specific phospholipase C (PI-PLC) which generates IP_3 and diacylglycerol (DAG). IP_3 is thought to act on sarcoplasmic reticulum (SR) IP_3 receptors inducing release of internal stores of

calcium which sequentially activates calmodulin and myosin light chain kinase. Recent studies indicate that this pathway is not involved in mediating contraction because complete suppression of IP_3 production with the PI-PLC inhibitor U 73122 has no effect on cholinergic contractions of the rat (Schneider et al., 2004b) or human (Schneider et al., 2004a) bladder.

Results from many studies in gastrointestinal (Murthy and Makhlouf, 1997; Murthy et al., 2003a; Zhou et al.), airway (Yang et al., 1996; Sohn et al., 2000) vascular (Horowitz et al., 1996; Woodrum et al., 1999; Rembold et al., 2000; Komalavilas et al., 2001), bladder and uterine smooth muscle (Taggart et al., 1999) and cells transfected with muscarinic receptor subtypes (May et al., 1999; Strassheim et al., 1999; Wang et al., 1999; Rumenapp et al., 2001; Ruiz-Velasco et al., 2002; Murthy et al., 2003b) indicate that many signal transduction enzymes are involved in muscarinic receptor mediated contraction and are summarized in figure 1. Few if any of these previously published studies were designed to determine which muscarinic receptor subtype mediates which of these pathways or the interaction between the pathways activated by the individual receptor subtypes.

METHODS:

Materials: The following drugs or chemicals were obtained from the sources indicated. Sigma Chemical Company, St. Louis, Mo: carbachol. Biomol, Plymouth Meeting, PA: ET-18-OCH_3 (1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine), D609 (O-Tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt), Y-27632 ((R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride), HA-1077 (1-(5-Isoquinolinesulfonyl)-homopiperazine·HCl), chelerythrine (1,2-Dimethoxy-N-methyl(1,3)benzodioxolo(5,6-c)phenanthridinium chloride), H7 ((1-(5-Isoquinolinesulfonyl)-2-methylpiperazine·2HCl) and H89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl). Darifenacin was a generous gift from Pfizer Limited (Sandwich, Kent).

Muscle Strips: Urinary bladders were removed from rats euthanized by CO_2 asphyxiation. The urinary bladder body (tissue above the ureteral orifices) was dissected free of the serosa and surrounding fat. The bladder was divided in the mid-sagittal plane, and cut into longitudinal smooth muscle strips (approximately 3 mm x 8 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 NaH_2PO4, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 23.8 mM $NaHCO_3$, and 5.6 mM glucose) and equilibrated with 95/5% O_2/CO_2 at 37 ^oC.

Carbachol Concentration Response: Following equilibration to the bath solution for 30 minutes, a maximal contraction induced by a 3 minute exposure to 120 mM potassium was recorded. The strips were ranked based on their contractile response to KCl and sorted so that

the average response in each treatment group was equal. The strips were incubated for 30 minutes in the presence or absence of an enzyme inhibitor and in the presence or absence of 30 nM darifenacin. Because higher doses of darifenacin appeared insurmountable and lower doses did not produce a significant shift in the concentration response curve, a single dose of 30 nM darifenacin was used. Dose response curves were derived from the peak tension developed following cumulative addition of carbachol (10 nM to 300 μM final bath concentration) and normalized to the response to 120 mM KCl. Only one concentration of enzyme inhibitor and or darifenacin was used for each muscle strip.

The target enzymes and K_i for the enzyme inhibitors are listed in table 1. The 3 different enzyme inhibitor concentrations used were based on these concentrations reported for isolated, purified enzymes starting at just above the published Ki and increasing at half log intervals. Dose 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 %, darifenacin affinity is based on EC_{25} values. The EC_{25} and EC_{50} values were determined for each strip using a sigmoidal curve fit of the data (Origin, Originlab Corp. Northampton, MA.). The EC_{25} values determined in the presence of darifenacin were used to estimate the pK_b and the dose ratios were determined using the same concentration of inhibitor with and without darifenacin. The estimated pK_b for darifenacin was calculated using the formula pK_b = -(log[darifenacin concentration]-log(dose ratio-1).

Because there were statistically significant differences in the variance between groups, statistical analysis of multiple group comparisons was performed by non-parametric Kruskal-

Wallis analysis of variance followed by a post hoc Mann-Whitney U test for pair wise comparisons (GB-STAT, Dynamic Microsystems, Silver Spring, MD). Because there was no statistical difference between the outcome measures for the different vehicle control groups, these data were pooled. For maximal contraction and carbachol potency, groups with enzyme inhibitors were compared to these pooled, no inhibitor added, vehicle controls.

RESULTS:

Inhibition of Phospholipases: Inhibition of PI-PLC with ET-18-OCH_3 has no effect on the carbachol stimulated maximal contraction response. A representative graph of the data for ET-18-OCH_3 is shown in figure 2 and table 3 includes this and data for all of the enzyme inhibitors used. Inhibition of PI-PLC with both 30 μM and 100 μM ET-18-OCH_3 decreases carbachol potency. Furthermore, when the strips were exposed to the highest concentration of ET-18-OCH_3 (100 μM), the affinity of darifenacin for inhibiting contraction was significantly lower than in strips exposed to vehicle only. Inhibition of PC-PLC with 100 μM D609 reduces the maximal contraction with no effect on carbachol potency or darifenacin affinity. Lower concentrations of D609 have no significant effects. The non-specific PLC inhibitor neomycin has only minor effects: a statistically significant reduction in the maximal contraction at the lowest concentration used with no effect at higher concentrations.

Inhibition of protein kinases: Inhibition of ROCK with 3 μM and 10 μM Y-27632 reduces carbachol potency with no effect on the maximal contraction. Additionally, darifenacin affinity is significantly increased following ROCK inhibition with both 1 μM and 10 μM Y-27632. Inhibition of ROCK along with PKG and PKA by all concentrations of HA-1077 decreases the carbachol maximal contraction and carbachol potency and at the same time decreases darifenacin affinity. Inhibition of PKC with chelerythrine has no effect on the maximal contraction response or carbachol potency. Inhibition of PKC does significantly increase darifenacin affinity. Inhibition of ROCK, PKA, PKC and PKG by all concentrations of H7 reduces the maximal carbachol contraction and carbachol potency. The 2 highest concentrations

of H7 (3 and 10 μM) increase darifenacin affinity. Inhibition of PKA, ROCK, and PKG with both 3 μM and 10 μM H89 reduces the maximal carbachol contraction while all concentrations used reduce carbachol potency. H89 has no effect on darifenacin affinity.

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DISCUSSION:

Our earlier studies demonstrated that the M_2 receptor subtype participates in mediating contraction from several experimental pathological conditions which result in hypertrophy (Braverman et al., 1998b; Braverman et al., 2000; Braverman et al., 2002; Braverman and Ruggieri, 2003). Because the M_2 subtype preferentially couples to $G_{\alpha i}$, while the M_3 subtype preferentially couples to $G_{\alpha q}$, this led to the hypothesis that the signal transduction pathways activated by the two receptor subtypes are different and may act in parallel to mediate contraction. Darifenacin is an M_3 selective muscarinic antagonist with about a 30 fold selectivity for M_3 over M_2 receptors. The affinity of darifenacin for inhibiting carbachol stimulated contraction is high in normal bladders consistent with the M_3 receptor subtype mediating contraction (Braverman and Ruggieri, 2003). A change in affinity of darifenacin to lower values induced by a given enzyme inhibitor suggests involvement of that enzyme in a signal transduction pathway mediated by the M_3 receptor. On the other hand, if an enzyme inhibitor increases the affinity of darifenacin, this may suggest the involvement of the inhibited enzyme in a parallel signal transduction pathway mediated by the M_2 receptor. Decreasing the maximal agonist induced contraction or the agonist potency with no change in darifenacin affinity could suggest the involvement of that enzyme in both M_2 and M_3 mediated pathways. A summary of our findings implicating the different possible signal transduction enzymes in M_2 and M_3 mediated contractile pathways is shown in table 4.

The PI-PLC inhibitor ET-18-OCH_3 reduces carbachol potency and darifenacin affinity indicating the possible involvement of PI-PLC in mediating the M_3 contractile signal. This

suggests that the M_2 receptor subtype has a greater role in mediating contraction following PI-PLC inhibition. Because D609 inhibits maximal contraction with no effect on darifenacin affinity, this suggests that PC-PLC is activated by both M_2 and M_3 receptors. Since the contraction following PC-PLC inhibition is mediated by the M_3 receptor subtype, this suggests that PC-PLC is essential for maximal force generation. Similar findings have been reported in the cat lower esophageal sphincter (Biancani et al., 1994). In permeabilized, isolated cat detrusor smooth muscle cells, inhibition of contraction with antibodies to G-proteins and PI-PLC isoforms indicates that cholinergic contraction occurs by M_3 activation of $G_{\alpha q/11}$, PI-PLC-1 and IP_3-dependent calcium release (An et al., 2002). Studies using M_2 and M_3 knockout mice indicate that M_2 receptors mediate bladder contraction indirectly by inhibition of adenylyl cyclase (Ehlert et al., 2005), however, findings in isolated rabbit intestine circular smooth muscle cells indicate that M_2 receptors can activate contraction directly through $G\beta\gamma_{i3}$-dependent activation of PI-PLC-β3 (Murthy et al., 2003a).

Our data differs from other reports of PI-PLC mediating rat and human bladder contraction (Fleichman et al., 2004; Schneider et al., 2004a; Schneider et al., 2004b). These reports concluded that bladder contraction via M_3 receptors largely depends on calcium entry through nifedipine-sensitive channels and activation of ROCK whereas phospholipase D and store-operated calcium channels contribute in a minor way and phospholipase C or PKC do not seem to be involved. This is likely due to differences in experimental paradigms. In our paradigm, each muscle strip is exposed to one single agonist concentration response curve (CRC) in separate smooth muscle strips with and without the enzyme inhibitor. The previously published

studies performed 5 repetitive CRCs in the same smooth muscle strip with increasing concentrations of enzyme inhibitor. We found that following 5 successive carbachol CRCs, the affinity of the M_3 selective antagonist para-fluoro hexahydro siladifenadol was shifted to a lower value, consistent with M_2 receptors participating in the contraction (data not shown). Others have shown that the M_3 receptor subtype is subject to agonist induced desensitization (Tobin et al., 1992; Willets et al., 2001; Willets et al., 2002; Griffin et al., 2004). Therefore M_3 receptor desensitization may occur during the repeated agonist CRCs which could explain these differences.

Recently much attention has been focused on calcium sensitization induced by the small GTP binding protein rho (Taggart et al., 1999; Murthy et al., 2003a; Fleichman et al., 2004; Schneider et al., 2004a). Calcium sensitization refers to the ability of the muscle to generate the same contractile force with lower levels of intracellular calcium and is likely due to inhibition of MLC phosphatase. Smooth muscle contractile force is largely a function of the phosphorylation state of myosin light chain. This phosphorylation state is in a balance between the activity of myosin light chain kinase and myosin light chain phosphatase. Rho kinase has been shown to phosphorylate CPI-17 which dramatically increases the inhibitory activity of CPI-17 on MLC phosphatase activity (Koyama et al., 2000). Y-27632, a specific inhibitor of rho kinase (Uehata et al., 1997), reduces carbachol potency and increases darifenacin affinity. This suggests that the M_3 receptor subtype mediates contraction following inhibition of the rho pathway and that the rho pathway is activated by M_2 receptor activation. The finding that inhibition of the rho pathway in normal bladders results in decreased carbachol potency with no decrease in the

carbachol maximal contraction, suggests that this pathway is active in normal bladders, but maximal contraction is not critically dependent on inhibition of MLC phosphatase.

HA-1077 has about a 5 fold higher affinity for inhibition of ROCK than PKA and PKG (Nagumo et al., 2000). When PKA and PKG along with ROCK are inhibited by HA-1077 there is a decrease in carbachol potency, a decrease in the carbachol maximum and a decrease in darifenacin affinity. The reduction in carbachol potency is likely the result of ROCK inhibition, because the specific ROCK inhibitor Y-27632 reduces carbachol potency. However, ROCK inhibition alone has no effect on the carbachol maximum, thus inactivation of either PKA or PKG blocks the carbachol maximum. The darifenacin affinity is decreased following inhibition of PKA, PKG and ROCK by HA-1077, but is increased following inhibition of only ROCK with Y-27632. This suggests that when ROCK is inhibited, PKA or PKG becomes involved in the pathway activated by the M_3 receptor and mediates contraction with only a decrease in carbachol potency. Once all 3 of these enzymes are inhibited, contraction occurs via an M_2 mediated pathway independent of ROCK, PKA and PKG.

Inhibition of PKC by chelerythrine has no effect on the carbachol maximum or carbachol potency. However, the darifenacin affinity is increased following PKC inhibition which suggests that PKC is involved in the M_2 receptor contractile signal transduction pathway. There are at least 11 different isoforms of PKC (α, βI, βII, γ, δ, ε, η, θ, μ, ζ and λ) and the isoform mediating this M_2 contractile pathway in rat bladder is not known. Because PKCα has been reported to be relatively insensitive to inhibition by chelerythrine (Lee et al., 1998; Davies et al.,

2000), it is likely that isoforms other than PKCα mediate this M_2 receptor activated contractile signal transduction pathway in rat bladder.

H89 has about a 5 fold higher affinity for inhibiting PKA than ROCK and about a 10 fold higher affinity for inhibiting PKA than PKG (Leemhuis et al., 2002). H89 inhibits the carbachol maximum and carbachol potency with no effect on darifenacin affinity. Because inhibition of ROCK alone only results in a decrease in carbachol potency, the decrease in carbachol maximum is likely the result of inhibition of PKA following H89. This suggests that PKA may be involved in mediating the M_3 contractile signal.

H7 has a about a 6 fold higher affinity for inhibition of ROCK than PKA and about a 10 fold higher affinity for inhibiting ROCK than PKC and PKG (Uehata et al., 1997). H7 inhibits the maximum contraction and carbachol potency while increasing darifenacin affinity suggesting that following inhibition of these enzymes, the M_3 receptor mediates contraction, but the remaining contractile signal is insufficient to stimulate maximum force. These results, as well as the results with HA-1077 suggest that PKA is involved in mediating the M_3 contractile signal. Because the darifenacin affinity is increased following H7, the M_3 receptor predominately mediates contraction in the presence of H7. However, when ROCK, PKA and PKG are inhibited with HA-1077, the residual contraction is mediated by the M_2 receptor. This difference is likely because HA-1077 does not inhibit PKC while H7 does. This result, as does the chelerythrine result described above, suggests that PKC is involved in mediating the M_2 contractile pathway.

Our results suggest that in normal bladders, where the M_3 receptor subtype predominantly mediates contraction, PI-PLC, PC-PLC, ROCK, PKC, PKA and/or PKG are all involved in transducing the contractile signal. The M_2 receptor may activate ROCK in the normal rat bladder because darifenacin affinity is increased following ROCK inhibition. This demonstrates that although the M_3 receptor appears to predominate in mediating contraction, the M_2 receptor does participate in the contractile signal. The contractile pathways activated by muscarinic receptors in the rat urinary bladder are complex, with redundant signaling pathways which become active when other signaling systems are inhibited. This redundant mechanism includes a more dominant role for the M_2 receptor following M_3 receptor desensitization, which likely occurs following multiple exposures to agonist.

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FIGURE LEGENDS:

Figure1. Diagram of potential muscarinic receptor stimulated signal transduction mechanisms.

Figure 2. A. Cumulative carbachol concentration response curves of rat bladder contraction with and without the PI-PLC inhibitor ET-18-OCH_3. The maximal carbachol response in the absence of any inhibitor is 121±5 % of the response to 120 mM KCl (2.41±0.14 g) and the carbachol EC_{50} is 1.9±0.6 μM.. B. Effect of ET-18-OCH_3 on the maximal carbachol response. C. Effect of ET-18-OCH_3 on carbachol EC_{50}. No inhibitor (n= 41); 10 μM (n=18); 30 μM (n=4); 100 μM (n=4). For figures 2-9 * denotes $p<0.05$, ** denotes $p<0.01$ compared to no inhibitor.

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Table 1. K_i for inhibitors at various lipases (μM) .

Inhibitor	PI-PLC	PC-PLC
ET-18-OCH_3	5	
D609		94
Neomycin	5	5

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Table 2. K_i for inhibitors at various kinases (μM).

Inhibitor	PKA	PKG	PKC	ROCK
Y-27632	25		26	0.1
HA-1077	1.6	1.6		0.33
Chelerytherine			0.66	
H7	3	5.8	6	0.45
H89	0.05	0.5		0.27

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Table 3. Effect of enzyme inhibitors on normal rat urinary bladder contractions.

Enzyme Inhibitor	Target Enzymes	Concentration (μM)	Carbachol Maximam (% KCl)	Carbachol EC_{50} (μM)	Darifenacin Affinity (nM)
Vehicle			117.8±4.7	2.4±0.2	5.3±1.0
ET-18-OCH_3	PI-PLC	10	116.8±3.6	4.5±1.8	8.7±3.3
		30	110.5±4.8	12.7±3.2**	3.7±1.0
		100	102.4±2.8	32.7±16.9**	15.4±4.9*
D609	PC-PLC	10	113.8±6.3	2.8±0.8	4.2±0.9
		30	91.4±5.9	3.4±0.2	3.8±1.1
		100	50.1±9.5**	10.8±5.9	2.1±1.1
Neomycin	PLC	10	90.3±5.3**	1.3±0.1	3.1±1.2
		30	115.2±9.1	2.1±0.6	5.3±1.9
		100	124.7±15.2	1.6±0.5	1.1±0.5
Y-27632	ROCK	1	101.4±7.1	3.0±0.7	0.2±0.03**
		3	106.7±11.4	6.7±1.8**	0.7±0.3
		10	110.1±9.8	12.7±3.4**	0.3±0.1*
HA-1077	ROCK, PKA, PKG	10	73.2±5.3**	22.0±6.3**	9.8±3.2
		30	70.5±6.2**	16.4±9.7**	6.4±4.8
		100	47.2±10.4**	25.8±10.3**	20.4±3.8**
Chelerytherine	PKC	1	101.8±9.8	5.4±3.3	2.6±0.6
		3	114.8±11.4	2.0±0.5	0.3±0.04**
		10	128.5±17.7	2.4±0.2	0.3±0.1**
H7	ROCK, PKA, PKG, PKC	10	75.2±5.3**	17.3±5.4	10.7±4.9
		30	82.0±6.6**	8.0±2.1	0.3±0.1**
		100	57.6±9.7**	19.6±6.8	0.5±0.2**
H89	PKA, ROCK, PKG	1	142.5±12.1	5.6±1.4**	6.6±5.2
		3	100.4±11.3*	3.8±0.8*	3.1±1.4
		10	53.0±6.8**	13.5±4.6**	6.2±3.2

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Table 4. Summary of findings.

Enzyme	M_2 pathways	M_3 pathways
PI-PLC	ET-18-OCH_3 decreases carbachol potency	ET-18-OCH_3 decreases darifenacin affinity ET-18-OCH_3 decreases carbachol potency
PC-PLC	D609 decreases carbachol potency	D609 decreases carbachol potency
ROCK	Y-27632 decreases darifenacin affinity Y-27632 decreases carbachol potency HA1077 decreases carbachol maximum HA1077 decreases carbachol potency H7 increased darifenacin affinity H7 decreases carbachol maximum H89 decreases carbachol maximum H89 decreases carbachol potency	HA1077 decreases darifenacin affinity HA1077 decreases carbachol maximum HA1077 decreases carbachol potency H89 decreases carbachol maximum H89 decreases carbachol potency
PKA	H7 increases darifenacin affinity H89 decreases carbachol maximum H89 decreases carbachol potency	H89 decreases carbachol maximum H89 decreases carbachol potency
PKC	Chelerytherine increases darifenacin affinity H7 increases darifenacin affinity	
PKG	H89 decreases carbachol maximum H89 decreases carbachol potency	H89 decreases carbachol maximum H89 decreases carbachol potency

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Figure 1

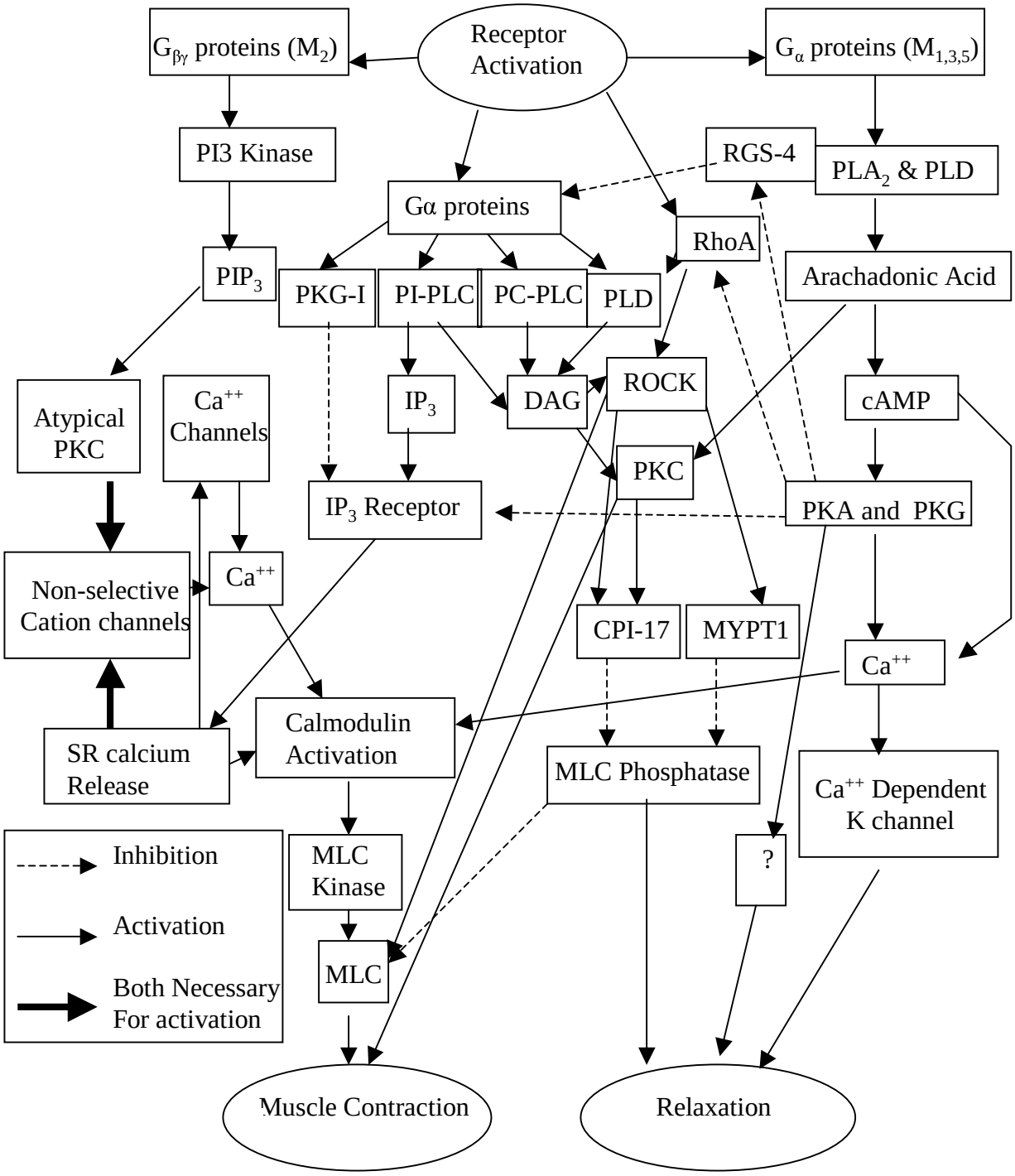

Figure 2