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Demonstration of functional M_3-muscarinic receptors in ventricular cardiomyocytes of adult rats

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1 Muscarinic receptors (M-receptors) in the mammalian heart are predominantly of the M_2-subtype. The aim of this study was to find out whether there might exist an additional myocardial non-M_2-receptor.

2 For this purpose, we assessed, in adult rat isolated ventricular cardiomyocytes, carbachol-induced [^{3}H]-inositol phosphate (IP) formation, and its inhibition by M-receptor antagonists.

3 Carbachol (10^{-7}–10^{-3} mol l^{-1}) increased IP-formation (maximal increase: $14\pm3\%$ above basal, $n=6$). This increase was significantly enhanced by pretreatment with pertussis toxin (PTX, 250 ng ml^{-1} for 20 h): maximal increase was $31\pm5\%$, pEC_{50}-value was 5.08 ± 0.33 ($n=6$).

4 In PTX-pretreated cardiomyocytes 100 μmol l^{-1} carbachol-induced IP-formation was inhibited by atropine (pK_i-value: 8.89 ± 0.10) and by the M_3-receptor antagonist darifenacin (pK_i-value: 8.67 ± 0.23) but was not significantly affected by the M_1-receptor antagonist pirenzepine (1 μmol l^{-1}) or the M_2-receptor antagonists AF-DX 116 and himbacine (1 μmol l^{-1}).

5 In conclusion, in adult rat cardiomyocytes there exists an additional, non-M_2-receptor, that is coupled to activation of the phospholipase C/IP_3-pathway; this receptor is very likely of the M_3-subtype.

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Abbreviations: DAG, diacylglycerol; IP, inositol phosphate; M, muscarinic; PLC, phospholipase C; PTX, pertussis toxin

Introduction

Muscarinic receptors (M-receptors) are subdivided into the five subtypes M_1, M_2, M_3, M_4 and M_5 (Caulfield & Birdsall, 1998). 'Odd' numbered M_1-, M_3- and M_5-receptors couple to a $G_{q/11}$-protein with subsequent activation of the phospholipase C/inositol trisphosphate/diacylglycerol (PLC/IP_3/DAG)-system, while 'even' numbered M_2- and M_4-receptors couple to a pertussis toxin (PTX) sensitive G-protein $G_{i/o}$ with subsequent inhibition of adenylyl cyclase activity. In the heart of various mammalian species, including humans, M_2-receptors are the predominant M-receptor subtype (Brodde & Michel, 1999; Dhein *et al*., 2001). However, during recent years evidence has accumulated that there might exist an additional, non-M_2-receptor, in the heart. Thus, in guinea-pig cardiac muscle (Ford *et al*., 1992) and in neonatal rat cardiomyocytes (Sun *et al*., 1996) muscarinic agonist-induced IP-formation was inhibited by M-receptor antagonists with an order of potency that did not fit with the characteristics of a M_2-receptor. Moreover, the existence of mRNA for M_1-receptors has been demonstrated in rat and guinea-pig ventricular cardiomyocytes (Sharma *et al*., 1996, 1997; Gallo *et al*., 1993). In addition, muscarinic agonists can evoke increases in IP-formation in rat and guinea-pig cardiomyocytes (Ford *et al*., 1992; Gallo *et al*., 1993; Sun *et al*., 1996; Colecraft *et al*., 1998); this is a typical response to stimulation of M_1-, M_3-, or M_5-receptors, but not of M_2- or M_4-receptors (see above).

The aim of this study, in isolated adult rat ventricular cardiomyocytes, was to further characterize the M-receptor subtype involved in carbachol-induced IP-formation. For this purpose, we studied the effects of the M-receptor antagonists pirenzepine (preferentially M_1-receptor antagonist), AF-DX 116 and himbacine (preferentially M_2-receptor antagonists), and darifenacin (preferentially M_3-receptor antagonist) on carbachol-induced IP-formation.

Methods

Preparation of cardiomyocytes

Left ventricular cardiomyocytes from 12-week-old male Wistar rats were isolated exactly as recently described (Pönicke *et al*., 2000). Freshly isolated cardiomyocytes were gently diluted in sterile culture medium M 199 pH 7.4 supplemented with 10% new-born calf serum. The resultant suspension of cardiomyocytes was transferred to 75 cm^2 cell culture flasks (3.6×10^4 cells cm^{-2}) and immediately used.

Inositol phosphate formation

The ventricular myocyte suspension was incubated for 20 h with myo-[^{3}H]-inositol (2.9 μCi ml^{-1}) at 37°C; in the majority of studies 250 ng ml^{-1} pertussis toxin (PTX) were added. We

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have recently shown that this concentration of PTX is sufficient to completely inactivate G_i-protein in rat cardiomyocytes (Pönicke *et al*., 1997). Thereafter, non-incorporated myo-[^{3}H]-inositol was washed out be centrifugation and the cells were resuspended in Hank's buffered saline solution supplemented with 10 mmol l^{-1} LiCl and 1% bovine serum albumin. Aliquots (970 μl) of the cardiomyocyte suspension (5×10^4 cells ml^{-1}) were incubated with carbachol in the presence and absence of M-receptor antagonists in a final volume of 1 ml for 45 min at 37°C. The antagonists atropine, pirenzepine, AF-DX 116, himbacine and darifenacin were added to the cardiomyocytes suspension 30 min before adding carbachol. Total [^{3}H]-inositol phosphates were isolated by Dowex AG1-X8 column chromatography exactly as recently described (Pönicke *et al*., 2000) and the radioactivity was determined in a liquid scintillation counter (Beckman LS 6000). Each data point was determined in quadruplicates.

Statistical evaluations

Data are means ± s.e.mean of n experiments. Experimental data for carbachol-induced [^{3}H]-inositol phosphate (IP) formation were analysed by fitting sigmoidal curves to the experimental data using the GraphPad Prism 3.0 program (GraphPad software, San Diego, CA, U.S.A.); in these calculations the bottom of the curves was fixed to 100% (i.e. no IP-formation above basal), the Hill-slopes were fixed to 1.0. Atropine and darifenacin concentration-inhibition curves were also analysed by the prism program 3.0.

Statistical significance of differences was estimated by non-paired two-tailed Student's t-test; a P-values <0.05 was considered significant. All statistical calculations were performed with the Prism program 3.0.

Drugs used

Myo-[^{3}H]-inositol (spec. Activity 4.25 TBq $mmol^{-1}$) was purchased from Amersham Buchler (Braunschweig, Germany), carbachol (carbamylcholine chloride) and atropine sulphate from Sigma-Aldrich (Deisenhofen, Germany), himbacine from Biomol Feinchemikalien GmbH (Hamburg, Germany) and pertussis toxin from Calbiochem (Bad Soden, Germany). Darifenacin hydrobromide (UK-088525-04) was kindly donated by Pfizer (Sandwich, Kent, U.K.), AF-DX 116 BS and pirenzepine dihydrochloride were kindly donated by Dr Karl Thomae GmbH (Biberach a.d. Riss, Germany). All other chemicals were of highest grade commercially available and obtained from sources recently described (Pönicke *et al*., 1999; 2000).

Results

Carbachol-induced [^{3}H]-Inositol phosphate formation

In isolated ventricular cardiomyocytes of adult rats carbachol (10^{-7}–10^{-3} mol l^{-1}) caused a concentration-dependent increase in [^{3}H]-inositol phosphate (IP) formation; maximal increase at 10^{-3} mol l^{-1} was 14 ± 3% above basal ($n=6$, $P<0.05$, Figure 1A); the pEC_{50}-value for carbachol was 5.01 ± 0.24 ($n=6$). Pretreatment of the cardiomyocytes with pertussis toxin (250 ng ml^{-1} for 20 h at 37°C) enhanced carbachol-induced [^{3}H]-IP formation: thus, in PTX-treated cardiomyocytes carbachol-induced [^{3}H]-IP formation was at each carbachol-concentration significantly higher than in non-treated cardiomyocytes (Figure 1A); maximal increase at 10^{-3} mol l^{-1} was 31 ± 5% above basal ($n=6$, $P<0.05$ *vs* the increase in non-treated cardiomyocytes), pEC_{50}-value for carbachol was 5.08 ± 0.33 ($n=6$).

Effects of muscarinic receptor antagonists on carbachol-induced [^{3}H]-inositol phosphate formation

Because of the higher response, all further experiments were performed in PTX-pretreated cardiomyocytes. Atropine

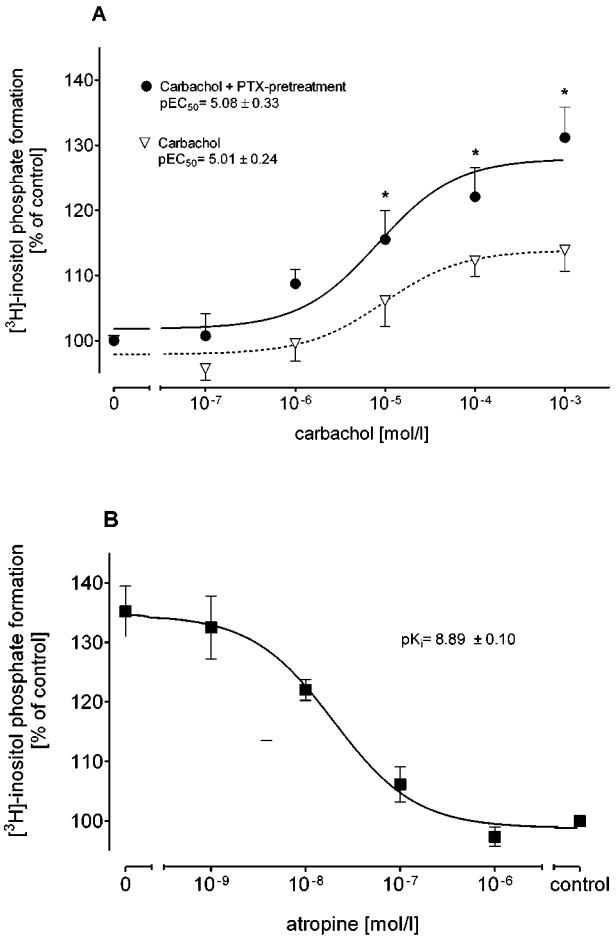

Figure 1 (A) Effects of pertussis toxin (PTX) pretreament (250 ng ml^{-1} for 20 h) on carbachol-induced [^{3}H]-inositol phosphate (IP) formation in adult rat ventricular cardiomyocytes. Ordinate: [^{3}H]-IP formation as percent of basal formation; abscissa: molar concentrations of carbachol. Basal [^{3}H]-IP formation was 1–2% of the incorporated radioactivity and amounted to 729 ± 72 d.p.m. in control, and to 729 ± 70 d.p.m. in PTX-treated cardiomyocytes. Means ± s.e.mean of six experiments, each performed in quadruplicates. *$P<0.05$ *vs* not-PTX-treated cells. (B) Effects of atropine on 100 μmol l^{-1} carbachol-induced [^{3}H]-IP formation in PTX-pretreated (250 ng ml^{-1} for 20 h) adult rat ventricular cardiomyocytes. Ordinate: [^{3}H]-IP formation as per cent of basal formation; Abscissa: molar concentrations of atropine. Basal [^{3}H]-IP formation was 1–2% of the incorporated radioactivity and amounted to 1061 ± 236 d.p.m.; 100 μmol l^{-1}-carbachol-induced [^{3}H]-IP formation was 1428 ± 306 d.p.m.; Means ± s.e.mean of three experiments, each performed in quadruplicates.

($10^{-9}-10^{-6}$ mol l^{-1}) concentration-dependently inhibited 100 μmol l^{-1} carbachol-induced [^{3}H]-IP formation (Figure 1B); at 10^{-6} mol l^{-1} atropine, carbachol-induced [^{3}H]-IP formation was completely suppressed. From these experiments a pK_i-value of 8.89 ± 0.10 ($n=3$) for atropine was calculated.

In contrast to atropine (non-subtype selective M-receptor antagonist) pirenzepine (1 μmol l^{-1}, antagonist preferentially at M_1-receptors), AF-DX 116 and himbacine (1 μmol l^{-1} each, antagonists preferentially at M_2-receptors) did not significantly affect 100 μmol l^{-1} carbachol-induced [^{3}H]-IP formation (Figure 2A). On the other hand, darifenacin (1 μmol l^{-1}, antagonist preferentially at M_3-receptors) completely suppressed 100 μmol l^{-1} carbachol-induced [^{3}H]-IP formation (Figure 2A).

In a final set of experiments we determined concentration-dependent inhibition of darifenacin ($10^{-9}-10^{-6}$ mol l^{-1}) in order to assess its affinity for the M-receptor involved in carbachol-induced [^{3}H]-IP formation. From the resulting concentration-inhibition curve (Figure 2B) a pK_i-value of 8.67 ± 0.23 ($n=5$) for darifenacin was calculated.

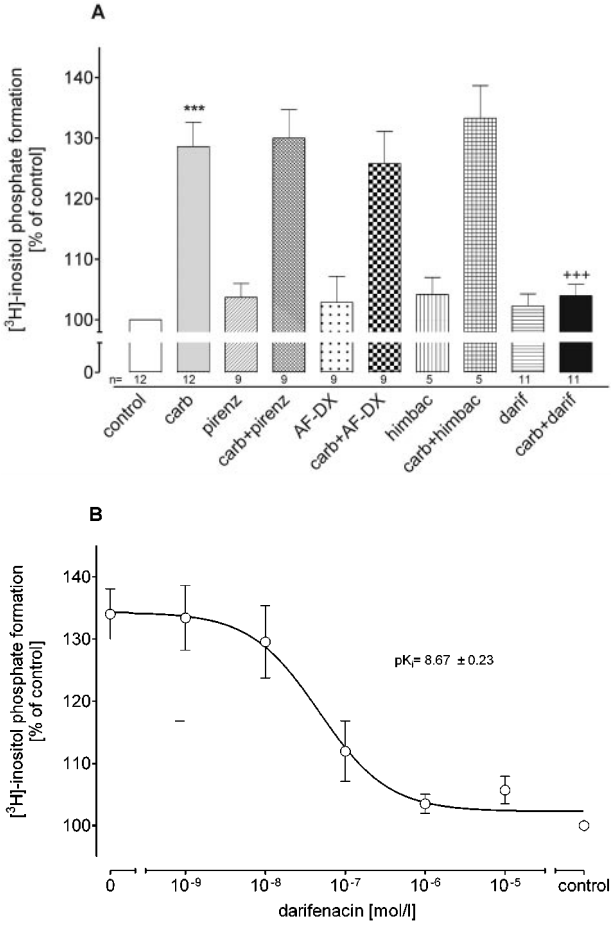

Figure 2 (A) Effects of pirenzepine (pirenz, 1 μmol l^{-1}), AF-DX 116 (AF-DX, 1 μmol l^{-1}), himbacine (himbac, 1 μmol l^{-1}) and darifenacin (darif, 1 μmol l^{-1}) on 100 μmol l^{-1} carbachol (carb)-induced [^{3}H]-inositol phosphate (IP) formation in PTX-pretreated (250 ng ml^{-1} for 20 h) adult rat ventricular cardiomyocytes. Ordinate: [^{3}H]-IP formation as percent of basal formation. Basal [^{3}H]-IP formation was 1–2% of the incorporated radioactivity and amounted to 805 ± 67 d.p.m. ($n=21$); 100 μmol l^{-1}-carbachol-induced [^{3}H]-IP formation was 1038 ± 371 d.p.m. ($n=21$); Means $\pm$s.e.mean; n=number of experiments, each performed in quadruplicates.***$P<0.01$ *vs* control, $+++P<0.01$ *vs* carbachol in the absence of darifenacin. Note, that none of the antagonists significantly affected basal [^{3}H]-IP formation. (B) Effects of darifenacin on 100 μmol l^{-1} carbachol-induced [^{3}H]-IP formation in PTX-pretreated (250 ng ml^{-1} for 20 h) adult rat ventricular cardiomyocytes. Ordinate: [^{3}H]-IP formation as percent of basal formation; Abscissa: molar concentrations of darifenacin. Basal [^{3}H]-IP formation was 1–2% of the incorporated radioactivity and amounted to 568 ± 65 d.p.m.; 100 μmol l^{-1}-carbachol-induced [^{3}H]-IP formation was 756 ± 82 d.p.m.; Means $\pm$s.e.mean of five experiments, each performed in quadruplicates.

Discussion

The main findings of this study were that, in adult rat ventricular cardiomyocytes, (a) carbachol induces IP-formation, and that is enhanced by pretreatment of the cells by PTX, and (b) the receptor involved is not an M_2-receptor but had the characteristics of an M_3-receptor.

Muscarinic receptors in the heart of various mammalian species are predominantly of the M_2-subtype (for recent review see Dhein *et al*., 2001). They couple *via* a PTX-sensitive G-protein to inhibition of adenylyl cyclase. Thus, IP-formation could be due to M_2-receptor mediated activation of G_i followed by release of the $\beta\gamma$-complex that can stimulate PLC resulting in increased IP-formation (for review see Wess, 1996).

Alternatively, however, IP-formation could be brought about by an 'odd' numbered M-receptor that couples to $G_{q/11}$ with subsequent activation of the PLC/IP_3-DAG-system.

In the present study the M-receptor subtype non-selective antagonist atropine inhibited carbachol-induced IP-formation with a pK_i-value of 8.9 which is well in its range of affinities to the various M-receptor subtypes (8.9–9.7, for reviews see Caulfield & Birdsall, 1998; Brodde & Michel, 1999). Thus, carbachol-induced IP-formation in rat ventricular cardiomyocytes is mediated by an M-receptor.

The preferentially at M_2-receptors acting antagonists AF-DX 116 and himbacine, however, did not affect carbachol-induced IP-formation. Both antagonists were used in a concentration (1 μmol l^{-1}) that occupies about 85–100% of M_2-receptors (Caulfield & Birdsall, 1998; Brodde & Michel, 1999); thus, if an M_2-receptor had been involved, the concentration of both antagonists would have been sufficient to inhibit IP-formation. Accordingly, it appears to be clear that M_2-receptors are not involved.

Similarly, the preferentially at M_1-receptors acting antagonist pirenzepine did not affect carbachol-induced IP-formation, although it was used in a concentration (1 μmol l^{-1}) that occupies nearly 100% of M_1-receptors (Caulfield & Birdsall, 1998; Brodde & Michel, 1999); this indicates that also M_1-receptors are not involved.

The preferentially at M_3-receptors acting antagonist darifenacin, however, inhibited carbachol-induced IP-formation with a pK_i-value (8.7) that fits very well in its range of affinity at M_3-receptors (8.4–8.9, Caulfield & Birdsall, 1998). Taken together, the high potency of darifenacin in combination with a lack of antagonistic effects of pirenzepine, AF-DX 116 and himbacine strongly supports the view that, in isolated adult rat ventricular cardiomyocytes, the M-receptor mediating carbachol-induced IP-formation is of the M_3-subtype.

It should be mentioned, however, that we measured IP-responses after culturing the cardiomyocytes for 20 h in medium supplemented with 10% serum. Thus, we cannot completely exclude the possibility that during this time the phenotype of the cardiomyocytes might have been changed. However, we believe that this is very unlikely for the following reasons: Eatman *et al.* (2000) have recently shown that, in chick cardiomyocytes, muscarinic receptor expression is stable for at least 72 h of incubation in medium supplemented with 10% serum. Moreover, we have recently demonstrated under identical conditions as in the present study that, in rat adult cardiomyocytes, noradrenaline evokes IP-formation predominantly *via* α_{1B}-adrenoceptor stimulation (Pönicke *et al.*, 2001) and this is in complete agreement with data obtained in rat ventricular slices after much shorter incubation times (Brodde & Michel, 1999); similarly, the prostanoid-receptor mediating IP-formation was identified in adult rat ventricular cardiomyocytes under the same incubation conditions as in the present study as FP-receptor and the same held true in rat ventricular slices after much shorter incubation times (Pönicke *et al.*, 2000); thus, for these two receptor subtypes the 20 h incubation in medium supplemented with 10% serum did not at all affect the phenotype; we therefore conclude that also in the present study phenotype of muscarinic receptor is not altered, although the final experimental proof is lacking.

Controversial data have been published in the literature as to whether or not the M-receptor subtype mediating in rat heart increases in IP-formation (and positive inotropic effects) is of the M_2- or of the M_1- or M_3-subtype. Thus, Sharma *et al.* (1996; 1997) have shown, using adult and neonatal rat ventricular cardiomyocytes, that carbachol causes increases in Ca^{2+} transients and IP-formation (Colecraft *et al.*, 1998). The increase in Ca^{2+} transients induced by high concentrations of carbachol (300 μmol l^{-1}) could be inhibited by pirenzepine in a rather low concentration (10 nmol l^{-1}) but not by M_2-receptor antagonist methoctramine (100 nmol l^{-1}); in addition, by single-cell RT–PCR mRNA for M_1- and M_2-receptors, but not for M_3- or M_4-receptors, could be detected. These data suggest that carbachol-induced increase in Ca^{2+}-transients is mediated by M_1-receptors, which is at variance with the present data. Unfortunately, these authors did not study the effects of various M-receptor antagonists on carbachol-induced IP-formation in order to classify the M-receptor subtype involved. Similarly, in ventricular cardiomyocytes of the guinea-pig the existence of M_1- (besides M_2-) receptors was demonstrated by RT–PCR (Gallo *et al.*, 1993). In these ventricular cardiomyocytes, too, carbachol-induced increases in Ca^{2+}-currents and IP-formation could be inhibited by pirenzepine in rather low concentrations (1 and 10 nmol l^{-1}). The reason for the discrepancy between our data and those published by Gallo or Sharma and co-workers is not known; however, it should be mentioned that in the studies of Gallo and Sharma and co-workers pirenzepine exhibited an extraordinary high affinity to the M_1-receptor (<1 nmol l^{-1}) that had not been seen in other studies. Moreover, Pappano and co-workers (Matsumoto & Pappano, 1991; Protas *et al.*, 1998; Shen *et al.*, 1999) have convincingly demonstrated that, in guinea-pig ventricular cardiomyocytes, only M_2-receptors are involved in muscarinic agonists-induced increases in Ca^{2+}-currents.

In contrast, Sun *et al.* (1996) demonstrated, in neonatal rat ventricular cardiomyocytes, that muscarinic receptor agonist induced IP-formation was antagonized by M-receptor antagonists with an order of potency HHSiD $>>$ pirenzepine $>$ AF-DX 116 that is in favour of the involvement of M_3-receptors, which is in agreement with our data. Moreover, in guinea-pig and canine atrial myocytes it has been shown that choline modulates cardiac membrane repolarization by activating of a M_3-receptor and its coupled K^+-channel (Shi *et al.*, 1999a), and in chick atria and ventricle a M_3-receptor coupled to IP-formation has been identified (Gadbut & Galper, 1994). Furthermore, the expression of M_3- in addition to M_2-receptors, but not of M_1-receptors, has been demonstrated in human (Hellgren *et al.*, 2000; Oberhauser *et al.*, 2001) chick (Gadbut & Galper, 1994) and canine hearts (Shi *et al.*, 1999b,c).

Finally, in the present study carbachol caused only weak increases in IP-formation; this might be due to the fact, that in rat ventricle only about 3% of the total M-receptor population is of the M_3-subtype (Kreji & Tuek, 2002). Interestingly, the carbachol-induced IP-formation was significantly enhanced after PTX-pretreatment of the cardiomyocytes, an effect that we have recently also observed in neonatal cardiomyocytes (Pönicke *et al.*, 1999). Moreover, in neonatal rat cardiomyocytes also endothelin-1 induced IP-formation *via* ET_A-receptor is enhanced by PTX-pretreatment (Pönicke *et al.*, 1997). On the other hand, Sun *et al.* (1996) could show that, in neonatal rat cardiomyocytes, pretreatment with PTX completely prevented carbachol-induced inhibition of adenylyl cyclase. These data are in favour of the idea that carbachol-induced IP-formation does not involve a PTX-sensitive G-protein; however, it appears that carbachol-induced IP-formation is under tonic inhibitory control of a PTX-sensitive G-protein. The mechanism underlying this tonic inhibition of $G_{q/11}$ coupled receptor mediated IP-formation by PTX sensitive G-proteins remains to be elucidated. In this connection it should be mentioned that also in non-PTX-treated cardiomyocytes carbachol-induced IP-formation was not affected by pirenzepine or AF-DX 116, but was completely blocked by atropine (data not shown). Thus it is unlikely that the PTX-treatment might have altered phenotype of the cardiomyocytes.

In conclusion: In adult rat cardiomyocytes carbachol-induced increase in IP-formation is enhanced by pretreatment of the cells with PTX, and is inhibited by M-receptor antagonists with an order of potency atropine = darifenacin $>>$ pirenzepine = AF-DX 116 = himbacine. This order of potency strongly supports the view that, in adult rat ventricular cardiomyocytes, M_3-receptors are involved in muscarinic agonist-induced IP-formation.

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