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Coupling of M_2 muscarinic receptors to Src activation in cultured canine colonic smooth muscle cells

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Singer, Cherie A., Sa Vang, and William T. Gerthoffer. Coupling of M_2 muscarinic receptors to Src activation in cultured canine colonic smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 282: G61–G68, 2002; 10.1152/ajpgi.00100.2002.—The purpose of this study was to determine whether Src tyrosine kinases are one of the signaling intermediaries linking M_2 receptor stimulation to extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) in cultures of canine colonic smooth muscle cells (CSMC). RT-PCR studies demonstrate expression of multiple Src tyrosine kinases, including Src, Fyn, and Yes, in CSMC. Muscarinic stimulation of CSMC with 10 μM ACh results in a twofold increase in Src activity within 10 min but does not increase the activity of Fyn. Treatment with the M_2 antagonist AF-DX 116 (10 μM) blocks ACh-stimulated Src activation in primary CSMC cultures that express both M_2 and M_3 receptors and in first-passage CSMC cultures that express predominantly M_2 receptors. Alkylation of M_3 receptors with 100 nM *N,N*-dimethyl-4-piperidinyl diphenylacetate mustard has no effect on Src activity. Treatment with the pyrazolopyrimidine Src inhibitor PP1 (10 μM) or AF-DX 116 (10 μM) blocks ACh-stimulated ERK phosphorylation. Together these results indicate that M_2 receptors are coupled to Src tyrosine kinase and subsequent activation of ERK in cultured CSMC.

Src; tyrosine kinase; colonic smooth muscle; extracellular signal-related kinase; mitogen-activated protein kinase

ACETYLCHOLINE is the principle excitatory neurotransmitter of the parasympathetic nervous system, acting at both nicotinic and muscarinic receptors in the ganglionic synapse and at muscarinic receptors in the postsynaptic junction. In the colon, the actions of ACh at the postsynaptic junction mediate smooth muscle contraction through M_2 and M_3 muscarinic receptors. M_3 receptors, coupled to the heterotrimeric GTP-binding protein (G protein) G_q/G_{11}, stimulate phospholipase C and phosphatidylinositol turnover to trigger release of stored calcium and activate protein kinase C (37). The more abundant M_2 receptors, coupled to G_i/G_o, inhibit adenylate cyclase, activate nonselective cation currents, and sensitize the contractile system to calcium (17, 33). The events mediated by M_2 and M_3 receptor activation contribute to changes in myoplasmic calcium levels necessary to generate contractile force. However, the signaling pathways that mediate these events are not completely understood.

The transduction of extracellular signals through G protein-coupled receptors involves the activation of multiple protein kinase cascades. Although specific pathways vary with different G proteins, activation of Src tyrosine kinases appears to be one of the signaling events immediately following receptor activation (14). Src is the prototypical member of a large family of tyrosine kinases expressed in a variety of cell types. These enzymes are membrane-associated nonreceptor protein tyrosine kinases activated by diverse stimuli and capable of phosphorylating numerous cellular targets (3, 32). Although muscarinic stimulation of Src tyrosine kinases in colonic smooth muscle (CSM) has not been well described, Src has been studied in smooth muscle under a variety of other conditions. Inhibition of tyrosine phosphorylation appears to affect smooth muscle contraction mediated by growth factors in gastric smooth muscle (39) and ceramide in CSM (20). In vascular smooth muscle, Src appears to be necessary for angiotensin II-mediated extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) activation (22). In addition, Src is thought to activate voltage-dependent Ca^{2+} channels (34, 35) and to stimulate phospholipase C activity (29).

These observations led our laboratory to determine which members of the Src tyrosine kinase family are intermediary molecules coupling muscarinic receptor stimulation to downstream signaling events in cultured CSM cells (CSMC). We have examined the expression of multiple Src tyrosine kinases, including Src, Fyn, and Yes, in canine CSM and have demonstrated that muscarinic stimulation of cultured CSMC results in the activation of Src but not Fyn tyrosine kinase. Additional pharmacological studies suggest that activation of Src in cultured CSMC is mediated via M_2 but not M_3 muscarinic receptors. Previous work in our laboratory (6, 13) demonstrated that activation of ERK MAPK in intact CSM is mediated by M_2 receptor stimulation in intact CSM, and we present data supporting the hypothesis that Src tyrosine kinase couples M_2 receptor stimulation to ERK MAPK in cultured CSMC.

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MATERIALS AND METHODS

Materials. Genistein and 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1) were obtained from Calbiochem (La Jolla, CA). *N,N*-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) mustard was purchased from Research Biochemicals International (Natick, MA). AF-DX 116 was purchased from Tocris Cookson (Ellisville, MO). Tissue culture reagents, TRIzol, and SuperScript II reverse transcriptase (RT) were purchased from GIBCO-BRL (Rockville, MD). ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, and 5.35 μg/ml linoleic acid) was purchased from Collaborative Biomedical Products (Bedford, MA). *Thermus aquaticus* polymerase (*Taq*), RNAse H, and goat anti-rabbit IgG conjugated to alkaline phosphatase were purchased from Promega (Madison, WI). All other PCR reagents were purchased from Invitrogen (San Diego, CA). Oligonucleotides for muscarinic receptors and Src tyrosine kinases were synthesized by Bio-Synthesis (Lewisville, TX). 18S rRNA primers were purchased from Ambion (Austin, TX). Enolase was purchased from Boehringer Mannheim (Indianapolis, IN). ^{32}P-labeled ATP was purchased from ICN Biomedicals (Costa Mesa, CA). Goat anti-Src (c-Src N-16), mouse anti-Fyn (Fyn 15), and rabbit anti-Src (Src 2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal phospho-p42/p44 ERK MAPK antibody was purchased from New England Biolabs (Beverly, MA). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture. The circular muscle layer of proximal colon was isolated from adult mongrel dogs of either sex killed with pentobarbital sodium (45 mg/kg iv). The tissue was minced and enzymatically digested as previously described (16). Dispersed cells were recovered by centrifugation and resuspended in M199 supplemented with 10% newborn calf serum, 0.2 mM glutamine, and antibiotics on collagen-coated flasks. Experiments were performed with confluent cultures of primary cells (1°CSMC) or confluent cultures of first-passage cells (1P CSMC) after 24 h of serum deprivation in M199 supplemented with ITS+. These studies were approved by the Institutional Animal Care and Use Committee.

RNA isolation and RT-PCR. Total RNA was isolated using TRIzol reagent from canine colonic circular smooth muscle (1 ml TRIzol/100 mg) and from confluent cultures of 1°CSMC and 1P CSMC (1 ml TRIzol/cm^2) according to the manufacturer's instructions. First-strand cDNA synthesis was performed at 42°C from 2 μg of RNA using 250 ng of random hexamers, 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.125 mM dATP, dTTP, dGTP, and cCTP, and 1 unit of SuperScript II reverse transcriptase in a volume of 30 μl. Src tyrosine kinases were amplified by PCR in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer). The reaction mixture contained 60 mM Tris·HCl (pH 8.5), 15 mM $(NH_4)SO_4$, 1.5 mM $MgCl_2$, 0.25 mM dATP, dCTP, dGTP, and dTTP, 0.2 ng/ml of each primer, template cDNA, and 1.25 units of *Taq*. Amplification of Src cDNA took place at 94°C for 1 min, 63°C for 30s, and 72°C for 30s for 35 cycles with oligonucleotides obtained from the mouse sequence (M17031): 5′-CCTTCCCGGCCTCACAGACACC-′3 and 5′-TCGGAGGGCGCCACATAGTGGC-′3. Amplification of Fyn cDNA took place at 94°C for 1 min, 57°C for 30 s, and 72°C for 30 s for 35 cycles using oligonucleotide derived from the human sequence (M14333): 5′-GGGCAGTTTGGGGAAGTATGGATG-′3 and 5′-TGGGGAACTTTGCACCTTGTCTTG-′3. Yes cDNA was amplified at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min for 35 cycles from oligonucleotides obtained from the canine spleen sequence (S81472): 5′-ATTTTCAGTGGTGCCAAGTCCATA-′3 and 5′-CACCCCTTATCTCATCCCAATCAC-′3. Amplification of M_2 and M_3 muscarinic receptor subtypes took place at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min using oligonucleotides obtained from the human sequence of the M_2 muscarinic receptor (X15264): 5′-CGGACCACAAAAATGGCAGGTA-′3 and 5′-TTGTATGGGGCCCAAGTGATGA-′3. Oligonucleotides for the M_3 muscarinic receptor were also obtained from the human sequence (U29589): 5′-AGCGTGGACGATGGAGGCAGTTT-′3 and 5′-TGGCACAGCAGCAGCATGTTGAA-′3. 18S rRNA was amplified using primers obtained from Ambion and was used as a control for cDNA synthesis and gel loading. Reaction products were separated by electrophoresis through a 1.2% agarose-Tris-acetate EDTA gel and visualized with ethidium bromide. Products were sequenced by dye terminator cycle sequencing on an ABI Prism 310 Genetic Analyzer (Perkin Elmer) according to the manufacturer's protocols.

Pharmacological studies in cultured 1P CSMC. Experiments were performed in confluent cultures of 1P CSMC serum deprived for 24 h. For time course experiments, cultures were stimulated with 10 μM ACh at the times indicated. In selected experiments, confluent cultures of 1P CSMC were preincubated for 15 min with a DMSO vehicle control (0.01%), the Src inhibitor PP1 (10 μM), the tyrosine kinase inhibitor genistein (10 μM), or diadzein (10 μM), an inactive congener of genistein. These inhibitors remained in the cultures during stimulation with 10 μM ACh for 10 min. In other sets of experiments, confluent cultures of 1P CSMC were preincubated for 15 min with the nonselective muscarinic receptor antagonist atropine (10 μM) or the M_2 receptor antagonist AF-DX 116 (10 μM). Whole cell lysates were prepared from all treatments by homogenization and centrifugation in RIPA [50 mM HEPES, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μM leupeptin, 0.5% Triton X-100, 0.5% NP-40, and 10% glycerol]. Protein concentrations were determined using the bicinchoninic acid method.

Immune complex assay for Src tyrosine kinase activity. Src or Fyn tyrosine kinase was immunoprecipitated from whole cell lysates by incubating 150 μg of total protein for 2 h with goat anti-Src (c-Src, N-16) in the presence of protein A/G agarose beads or with an agarose conjugate of mouse anti-Fyn (Fyn 15). These antibodies are kinase specific, and according to the manufacturer they do not cross-react with other known Src tyrosine kinases. In addition, preliminary experiments were performed to verify the specificity of these antibodies in our cultures (data not shown). Beads were then washed twice in RIPA and once in kinase assay buffer (50 mM PIPES, pH 7.0, 10 mM $MnCl_2$, and 10 mM DTT). Immunoprecipitates of either Src or Fyn tyrosine kinase were incubated for 60 min at 30°C in kinase assay buffer, 40 μCi of [γ-^{32}P]ATP, and 4 μg of acid-treated enolase according to published protocols (4, 7). Proteins were separated by 8% SDS-PAGE, enolase phosphorylation was detected by phosphorimaging, and densitometric analysis was performed (model 525 molecular imager; Bio-Rad, Hercules, CA). Aliquots from the immunoprecipitates were also separated by 8% SDS-PAGE and immunoblots performed with a rabbit anti-Src antibody (Src2). Although this antibody recognizes Src, Fyn, Yes, and Fgr tyrosine kinases, it is useful in monitoring the immunoprecipitation of individual kinases. Immunoreactive bands were visualized after incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. Images of immunoblots scanned with a UMAX Powerlook flatbed scanner were analyzed by densitometry, and enolase phosphorylation was normalized to the immunoreactivity measured from the immunoprecipitates.

Pharmacological studies with 4-DAMP mustard and AF-DX 116 in cultured primary CSMC. Experiments were performed in confluent cultures of 1°CSMC serum deprived for 24 h. 4-DAMP mustard was activated in M199 supplemented with ITS+ at 37°C for 30 min. Cultures were then incubated for 30 min with 100 nM 4-DAMP mustard in the presence of 1 μM AF-DX 116 to alkylate M_3 receptors while protecting M_2 receptors. Activated, unbound 4-DAMP mustard was inactivated by incubating the treated cultures for 15 min in fresh medium containing 0.5 mM $Na_2S_2O_3$ and 1 μM AF-DX 116. Control cultures received 0.1% DMSO and were also treated with 0.5 mM $Na_2S_2O_3$ and 1 μM AF-DX 116. Cultures were then stimulated with 10 μM ACh for 10 min, and whole cell lysates were prepared for immune complex kinase assays. Details of this protocol were described previously (6, 11).

ERK MAPK immunoblots. Samples from 1P CSMC generated to measure Src tyrosine kinase activity were also used to examine ERK MAPK phosphorylation. Ten micrograms of total protein from whole cell lysates were separated by 10% SDS-PAGE. Immunoblots were incubated with the phospho-p44/p42 ERK MAPK primary antibody (1:1,000). This antibody recognizes phosphorylation of the tyrosine and threonine residues in p44 ERK1 and p42 ERK2 MAPK necessary for activation of these enzymes. Results were visualized after incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000), and densitometry was performed as described.

Statistical analysis. Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA). A two-tailed Student's *t*-test was used to test unpaired parametric data, and a Mann-Whitney rank-sum test was used to test nonparametric data. The null hypothesis was rejected if $P < 0.05$.

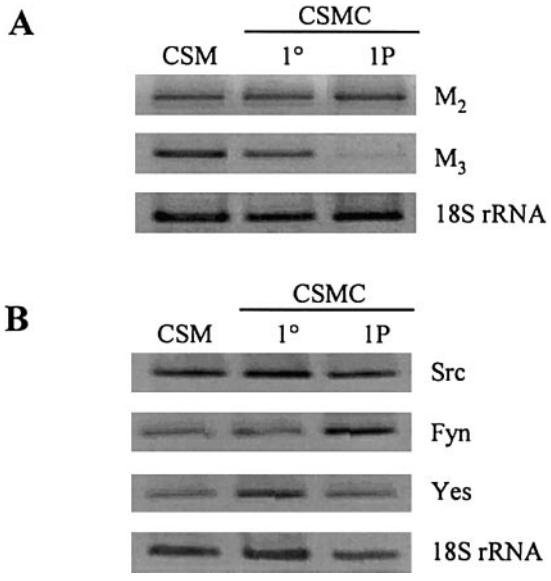

Fig. 1. Expression of muscarinic receptors and Src tyrosine kinases in colonic smooth muscle (CSM) and cultured colonic smooth muscle cells (CSMC). Total RNA samples were prepared from intact canine CSM, primary cultures of CSMC (1°CSMC), or first-passage cultures of CSMC (1P CSMC). First-strand cDNA synthesis and PCR proceeded as described in MATERIALS AND METHODS. Amplification of 18S rRNA is shown as a control for cDNA synthesis and gel loading. *A*: qualitative RT-PCR demonstrating expression of M_2 and M_3 muscarinic receptors. *B*: qualitative RT-PCR demonstrating expression of Src, Fyn, and Yes tyrosine kinases. Results were duplicated in 3 separate CSM and CSMC preparations. Data shown are from a representative experiment.

RESULTS

Radioligand binding and quantitative PCR studies have established the expression of M_2 and M_3 receptors in canine circular smooth muscle (6, 38). Figure 1*A* presents qualitative RT-PCR studies performed in CSMC cultures to compare M_2 and M_3 receptor expression with that found in intact CSM. Reaction products of the expected size were amplified from CSM, cultured 1°CSMC, and 1P CSMC with receptor-specific primers and verified by sequencing. M_2 receptor (810 bp; Fig. 1*A*, *top*) and M_3 receptor (450 bp; Fig. 1*A*, *middle*) expression is shown along with RT-PCR amplification of 18S rRNA (324 bp, Fig. 1*A*, *bottom*), which is used as a control for cDNA synthesis and gel loading. As expected, M_2 and M_3 receptors are expressed in CSM. Cultured 1°CSMC retain expression of M_2 and M_3 receptors, but after passage of the cells and further time in culture, 1P CSMC appear to predominantly express M_2 receptors.

To address the issue of which Src tyrosine kinases may be expressed in CSM, qualitative RT-PCR was again performed in CSM and cultured CSMC. Although multiple Src tyrosine kinases have been cloned from a variety of mammalian cell types, we chose to concentrate our efforts on Src, Fyn, and Yes, three enzymes ubiquitously expressed in most tissues. Figure 1*B* shows Src-family tyrosine kinase expression in CSM, 1°CSMC, and 1P CSMC using gene-specific primers. All reactions amplified single PCR products of the expected size with nucleotide identities of 89% to mouse neuronal Src, 93% to human placental Fyn, and 94% to canine splenic Yes. Translation of the sequenced products revealed 93%, 96%, and 98% identity to Src, Fyn, and Yes, respectively. The results suggest that more than one Src tyrosine kinase is expressed in CSM, which is consistent with data obtained from cultured vascular smooth muscle (9). In addition, CSMC appear to retain expression of Src, Fyn, and Yes with time in culture.

To determine which of the Src tyrosine kinases present in CSMC are activated by a muscarinic stimulus, immune complex kinase assays were performed using acid-treated enolase as a substrate for tyrosine phosphorylation. The method allows for the separation of individual kinases from whole cell lysates by immunoprecipitation of the kinase of interest. In the described experiments, 1P CSMC were serum deprived for 24 h and treated at the times indicated in Fig. 2 with 10 μM ACh. Control experiments were initially performed with 1P CSMC cultures treated with 10 ng/ml platelet-derived growth factor (PDGF), which is a known stimulant of Src tyrosine kinases in non-muscle cells. PDGF stimulation of 1P CSMC increased tyrosine kinase activity of Src and Fyn immunoprecipitates in a time-dependent manner (data not shown). In contrast, ACh stimulation of 1P CSMC only increased the activity of Src immunoprecipitates, with peak activation of an approximately twofold increase over

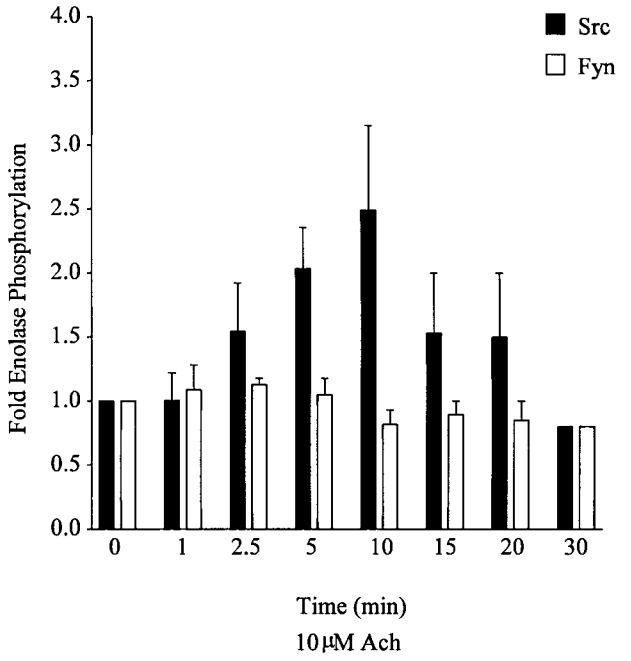

Fig. 2. Src and Fyn tyrosine kinase activity in CSMC. Cultures of 1P CSMC were serum deprived for 24 h before treatment with 10 μM ACh at the times indicated. Immune complex in vitro kinase assays were performed from Src or Fyn immunoprecipitates as described in MATERIALS AND METHODS. Enolase phosphorylation was normalized to the amount of tyrosine kinase present in the sample as determined by immunoblotting of the immunoprecipitates. Data are expressed as mean ± SE fold change relative to the absence of ACh at 0 min (n = 2–3).

basal levels after 10 min of ACh stimulation. These results suggest that, although multiple Src tyrosine kinases are expressed in intact canine CSM and cultured CSMC, Src, but not Fyn, tyrosine kinase is activated by a muscarinic stimulus. The specificity of acid-treated enolase as a substrate for Src in cultured CSMC was examined by treating cultured 1P CSMC with PP1, genistein, or diadzein in the presence of ACh. The effect of these drugs on Src tyrosine kinase activity in an immune complex kinase assay after Src immunoprecipitation is shown in Fig. 3, in which inhibition of tyrosine phosphorylation with 10 μM genistein or inhibition of Src with 10 μM PP1 blocks enolase phosphorylation in Src immunoprecipitates. Treatment with 10 μM diadzein, the inactive congener of genistein, did not affect enolase phosphorylation.

As shown in Fig. 1*A*, RT-PCR demonstrated that the 1P CSMC used in these experiments predominantly express the M_2 muscarinic receptor, thereby making it plausible that the observed increases in Src activity may be mediated via M_2 receptors. This was tested by examining the coupling of M_2 receptors to Src activation in the presence of the nonselective muscarinic receptor antagonist atropine and the M_2-specific antagonist AF-DX 116 (Fig. 4). Stimulation of Src tyrosine kinase activity with 10 μM ACh for 10 min in the presence of 10 μM atropine or 10 μM AF-DX 116 results in a significant decrease in Src activity. In addition, the presence of atropine does not further reduce Src activity beyond that seen with AF-DX 116, suggesting that M_2 receptors are the muscarinic receptors present in these cells that significantly contribute to activation of Src tyrosine kinase. This result, however, does not rule out the possibility that M_2 receptors mediate Src activity in the absence of M_3 receptors.

To confirm coupling of M_2 receptors to Src in CSMC, we utilized confluent cultures of 1°CSMC, which express both M_2 and M_3 receptors. Coupling of Src to M_2 receptors was investigated in 1°CSMC as described above for 1P CSMC, in which ACh-stimulated Src activity was measured in the presence of 10 μM AF-DX 116. Coupling of Src to M_3 receptors was investigated in 1°CSMC by alkylation of M_3 receptors with 100 nM 4-DAMP mustard in the presence of 1 μM AF-DX 116 with a protocol previously published by our laboratory (6). The inclusion of AF-DX 116 protects M_2 receptors during alkylation by 4-DAMP mustard, which is only modestly selective for M_3 receptors [dissociation constant (K_d) = 7.2 nM for M_3 receptors; K_d = 43 nM for M_2 receptors]. The results from these experiments are

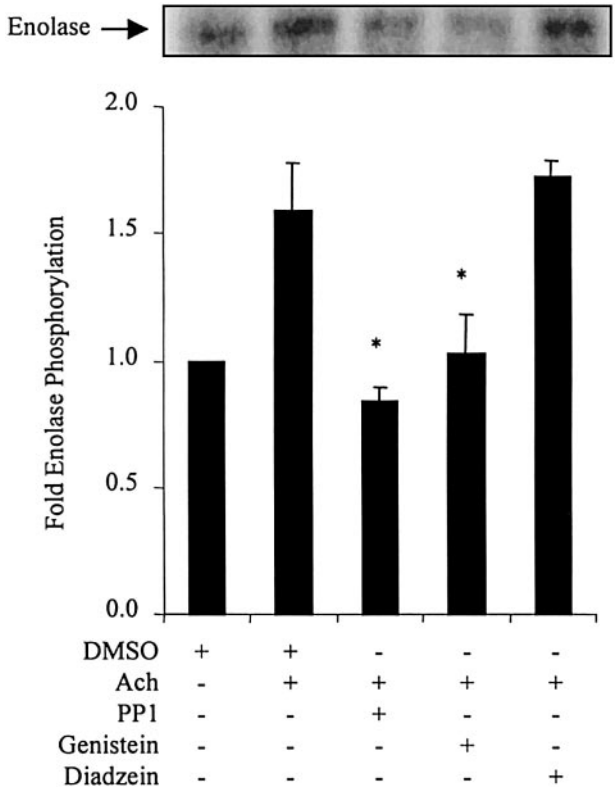

Fig. 3. Inhibition of Src tyrosine kinase activity by genistein and 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1). 1P CSMC were serum deprived for 24 h. Tyrosine kinase activity was stimulated on treatment with 10 μM ACh for 10 min in the presence of a DMSO vehicle control, 10 μM PP1, 10 μM genistein, or 10 μM diadzein. Immune complex in vitro kinase assays were performed as described for Src immunoprecipitates, and enolase phosphorylation was normalized as described in Fig. 2. Data are expressed as mean ± SE fold change in enolase phosphorylation relative to the DMSO control cultures (n = 3). A representative phosphorimage of enolase phosphorylation is shown along with a graphic summary of the data. *Significant difference from ACh-treated group, $P < 0.05$.

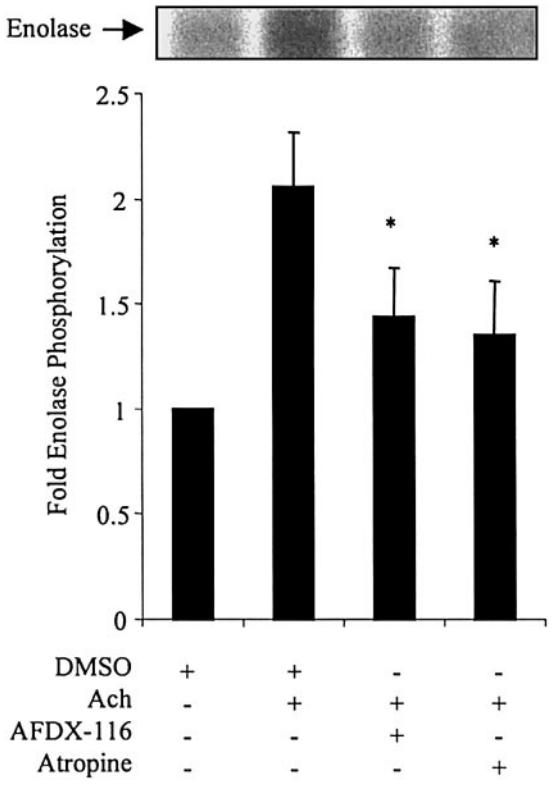

Fig. 4. Effects of AF-DX 116 and atropine on Src activity in 1P CSMC. Confluent cultures of 1P CSMC were serum deprived for 24 h. Src tyrosine kinase activity was stimulated on treatment with 10 μM ACh for 10 min in the presence of a DMSO vehicle control, 10 μM AF-DX 116, or 10 μM atropine. Immune complex in vitro kinase assays were performed from Src immunoprecipitates, and enolase phosphorylation was normalized as described in Fig. 2. Data are expressed as mean ± SE fold change in enolase phosphorylation relative to the DMSO control cultures (n = 6). A representative phosphorimage of enolase phosphorylation is shown along with a graphic summary of the data, *Significant difference from ACh-treated group, $P < 0.05$.

presented in Fig. 5, in which treatment with 10 μM AF-DX, but not 100 nM 4-DAMP mustard, blocks ACh-stimulated Src activity. Doses of 1 or 10 nM 4-DAMP mustard produced similar results (data not shown). These results suggest that in the presence of M_2 and M_3 receptors, M_2 receptors are coupled to Src tyrosine kinase activity in cultured CSMC.

Previous work from our laboratory (6) in intact canine CSM demonstrated that M_2 receptor activation is coupled to ERK MAPK phosphorylation. In 1P CSMC, ERK phosphorylation peaks after 10 min of ACh stimulation and is sustained above basal levels for at least 30 min (data not shown), consistent with previous reports from our laboratory (13) in intact canine CSM. To determine whether Src tyrosine kinase is one of the signaling intermediaries coupling M_2 receptors to ERK, we again used the Src inhibitor PP1 and measured ERK phosphorylation from samples in which Src activity had been previously determined (Fig. 6*A*). In these samples, 10 μM PP1 blocks both ACh-stimulated ERK1 and ERK2 phosphorylation, establishing that Src tyrosine kinase is an upstream activator of ERK after a muscarinic stimulus in 1P CSMC. Figure 6*B* demonstrates that treatment with 10 μM AF-DX 116 blocks ACh-stimulated ERK phosphorylation, thereby confirming the results reported from intact tissue (6) in cultured CSMC. Together these results clearly demonstrate that Src tyrosine kinase couples M_2 receptor activation to ERK MAPK phosphorylation in cultured CSMC.

DISCUSSION

Although contraction of gastrointestinal smooth muscle is mediated primarily through M_3 receptors (10), which are directly coupled to increases in intracellular calcium concentrations, the functional role of M_2 receptors in smooth muscle remains unclear. The absence of M_3 receptors on alkylation with 4-DAMP mustard results in M_2 receptors mediating a majority of the contractile response in guinea pig trachea, but the same is not true in the guinea pig esophagus or

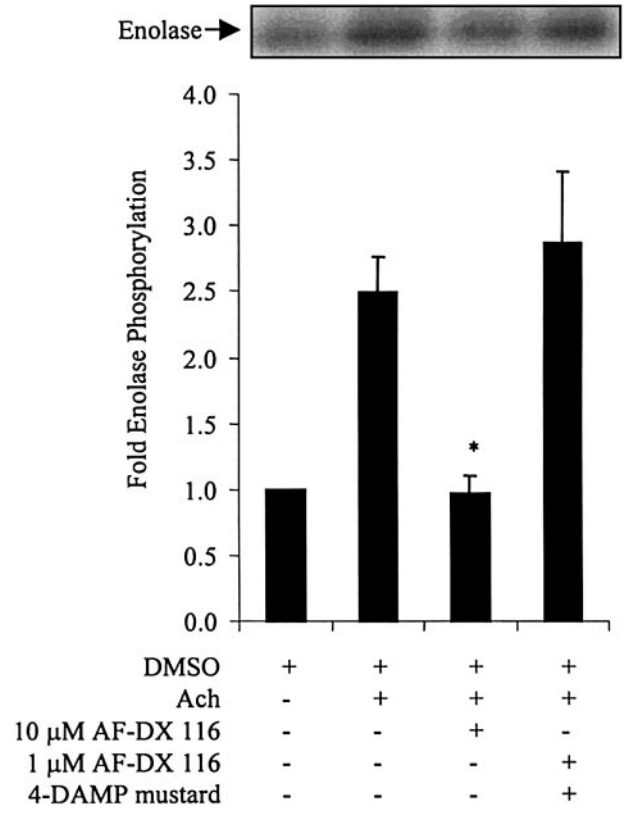

Fig. 5. Effects of AF-DX 116 and *N*,*N*-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) mustard on Src activity in 1°CSMC. Confluent cultures of 1°CSMC were serum-deprived for 24 h. M_2 receptors were inhibited by treatment with 10 μM AF-DX 116. M_3 receptors were alkylated as described in MATERIALS AND METHODS with activated 4-DAMP mustard (100 nM) in the presence of 1 μM AF-DX 116. Control cultures received 0.1% DMSO and were stimulated with 10 μM ACh for 10 min. Whole cell lysates were prepared and immune complex kinase assays performed from Src immunoprecipitates. Enolase phosphorylation was normalized as described in Fig. 2. Data are expressed as mean ± SE fold change in enolase phosphorylation relative to the DMSO control cultures (n = 2–3). A representative phosphorimage of enolase phosphorylation is shown along with a graphic summary of the data. *Significant difference from ACh-treated group, $P < 0.05$.

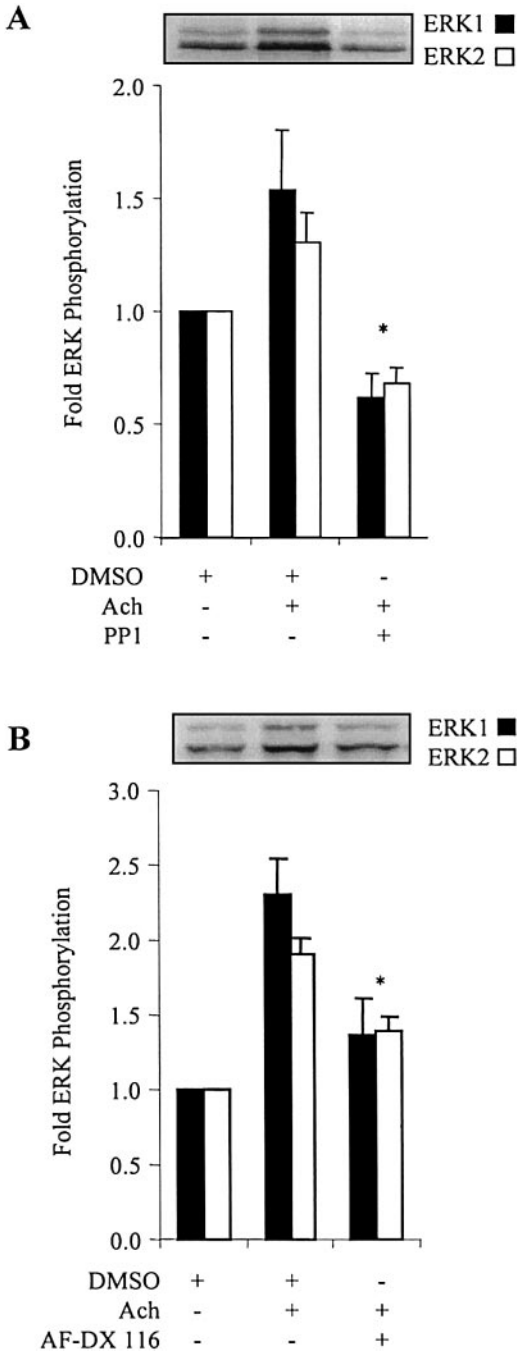

Fig. 6. Coupling of Src and M_2 receptors to ACh-stimulated extracellular signal-related kinase (ERK) mitogen-activated protein kinase (MAPK) phosphorylation. 1P CSMC were grown to confluence and serum deprived for 24 h before stimulation with 10 μM ACh for 10 min. Aliquots (10μg) of whole cell lysates were separated by SDS-PAGE, and immunoblots were performed to examine phosphorylation of ERK1 and ERK2 MAPK. *A*: effect of 10μM PP1 on ACh-stimulated ERK MAPK phosphorylation; values are means ± SE (n = 3). *B*: effects of 10 μM AF-DX 116 on ACh-stimulated ERK MAPK phosphorylation; values are means ± SE (n = 6). Representative immunoblots are shown along with graphic summaries. *Significant difference from ACh-stimulated group, $P < 0.05$.

colon (31). Previous studies in our laboratory (6) extended these results in canine colon, in which M_2 receptors appear to play only a minor role in directly mediating contraction. It is interesting to note, however, that the ratio of M_2 to M_3 receptors differs in various smooth muscle preparations. In the circular smooth muscle of canine colon, radioligand binding studies and quantitative PCR methods have determined that M_2 receptors are more abundant than M_3 receptors (6, 38). In the CSM culture model presented in our studies, the expression of M_2 and M_3 receptors differs in culture from that seen in intact tissue. Confluent cultures of primary CSMC express both M_2 and M_3 receptors, but with time in culture, 1P CSMC predominantly express the M_2 receptor subtype. The functional implication of this result may be explained by work done in airway smooth muscle (15). In first-passage airway smooth muscle cultures, Halayko et al. (15) described limited expression of M_3 receptors. Prolonged serum deprivation results in an upregulation of M_3 receptors along with an increase in smooth muscle α-actin, smooth muscle myosin heavy chain, and SM22, a smooth muscle-specific actin-binding protein, in a subpopulation of cells. More importantly, these cultures acquire a functional contractile phenotype. Given these observations, the absence of M_3 receptors in our 1P CSMC suggests that these cells do not retain a contractile phenotype. These cultures do provide a nice model system in which to study M_2 receptor-mediated signaling events, which may indirectly contribute to the contractile response through signaling pathways that affect cAMP production, nonselective cation channels, or calcium sensitization of contractile proteins.

Very few data exist on the effects of muscarinic stimulation on tyrosine phosphorylation in smooth muscle, but there is a growing body of evidence on the effects of growth factor stimulation in smooth muscle, which suggests that M_2 receptor-mediated events may activate tyrosine kinases. Growth factors that increase tyrosine phosphorylation, such as epidermal growth factor and PDGF, lead to contraction of gastric and vascular smooth muscle preparations (1, 2, 36). Contraction induced by these growth factors can be blocked with tyrosine kinase inhibitors such as genistein and tyrphostin (28, 36). In addition, prolonged tyrosine phosphorylation due to treatment with protein tyrosine phosphatase inhibitors such as vanadate stimulates contraction (8, 19, 25). In vascular smooth muscle, the tyrosine kinase inhibitor genistein decreases contraction, possibly by blocking calcium influx (34). Genistein also blocks agonist-induced sensitization of contractile proteins to calcium in ileal smooth muscle (30). In CSM, the tyrosine kinase Src modulates voltage-dependent calcium channel activity induced by PDGF (18) and ceramide-stimulated Src activation is reduced in the absence of calcium (20).

The Src tyrosine kinases are a large family of nonreceptor tyrosine kinases activated by multiple signals with varying tissue expression patterns. In vascular smooth muscle, Src tyrosine kinase is expressed (24),

whereas other members of the Src family, including Fyn, Lyn, Hck, and Fgr, are expressed in cultured human aortic smooth muscle cells (9). The data presented here demonstrate that Src, Fyn, and Yes tyrosine kinases are expressed in CSM and cultured CSMC. The functional role of multiple Src tyrosine kinases in CSM is not known, and with such a large gene family of tyrosine kinases it is difficult to assess the contribution of each enzyme. Therefore, in these studies, we chose to concentrate our efforts on Src and Fyn tyrosine kinases. On muscarinic stimulation of CSMC, however, only Src tyrosine kinase activity was increased. Because both Src and Fyn activities are increased with PDGF stimulation in these cultures, it appears that Fyn is an active kinase but is not coupled to muscarinic stimulation as measured in these studies. These results suggest that tyrosine kinases may be differentially coupled to a variety of stimuli in CSM and that Src is one of the tyrosine kinases coupled to muscarinic receptor signaling.

The signaling intermediates linking pertussis toxin-sensitive G_i-coupled receptors to Src activation have been elucidated in nonmuscle cells. On stimulation, $G\beta\gamma$-subunits are liberated from the G protein complex and promote assembly of signal transduction complexes consisting of Src, Shc, and Grb2 proteins. Activation of this signaling complex goes on to mediate Ras-dependent activation of MAPK (26). Previous work in our laboratory (6, 13) demonstrated that muscarinic stimulation induces ERK MAPK activation in colonic smooth muscle. Alkylation of M_3 receptors with 4-DAMP mustard did not affect ERK MAPK phosphorylation, but inhibition of M_2 receptors with AF-DX 116 blocked muscarinic stimulation of ERK, leading to the conclusion that this effect is mediated via M_2, but not M_3, receptors. In light of the data presented here, it is likely that Src tyrosine kinase may be an upstream mediator of M_2 receptor-mediated signaling in CSM. AF-DX 116 blocks M_2 receptor stimulation of Src in 1P CSMC cultures that express M_2 receptor and in 1°CSMC that express both M_2 and M_3 receptors, whereas alkylation of M_3 receptors does not affect muscarinic stimulation of Src activity. In addition, inhibition of Src with PP1 or treatment with AF-DX 116 blocks ACh-induced ERK MAPK phosphorylation. Thus Src appears to be the intermediary signaling molecule coupling M_2 receptors to ERK MAPK. This is supported by previous results in vascular and airway smooth muscle, in which angiotensin II- and PDGF-stimulated Src activation is necessary for ERK MAPK activation (5, 22). These results also concur with those found in nonmuscle cells, demonstrating that Src participates in M_2 receptor activation of ERK (21).

The functional significance of muscarinic stimulation of ERK MAPK in CSM remains to be defined. Our laboratory has proposed (6, 12) that ERK MAPK may contribute to calcium sensitization, possibly through phosphorylation of the actin-binding protein caldesmon, even though the role of caldesmon during contraction is not clear. It is also likely that Src-mediated activation of MAPK signaling pathways leads to the phosphorylation of numerous targets, which may be intimately involved in contractile processes and in remodeling of the actin cytoskeleton. Potential targets of this pathway include myosin light chain kinase (23) calponin (27), and elements of focal adhesion complexes that couple actin filaments to the cell membrane. In addition, there are numerous ERK MAPK substrates whose functions may play peripheral roles in regulating the response such as other kinases, transcription factors, and phospholipases. Therefore, although activation of M_2 receptors alone produces only a modest mechanical response in smooth muscles, there are numerous downstream targets for Src-mediated activation of ERK MAPK that could contribute to contraction, cytoskeletal structure, and regulation of pathways important in smooth muscle growth and phenotype determination.

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