Received: 6 October 2020 | Revised: 7 May 2021 | Accepted: 24 May 2021

DOI: 10.1096/fj.202002213R

RESEARCH ARTICLE

THE FASEB JOURNAL

M_1 and M_2 mAChRs activate PDK1 and regulate PKC βI and ε and the exocytotic apparatus at the NMJ

V. Cilleros-Mañé | **L. Just-Borràs** | **A. Polishchuk** | **M. Durán** | **M. Tomàs** | **N. Garcia** | **J. M. Tomàs** | **M. A. Lanuza**

Unitat d'Histologia i Neurobiologia (UHNEUROB), Facultat de Medicina i Ciències de la Salut, Departament de Ciències Mèdiques Bàsiques, Universitat Rovira i Virgili, Reus, Spain

Correspondence
J. M. Tomàs and M. A. Lanuza, Departament de Ciències Mèdiques Bàsiques, Universitat Rovira i Virgili, Unitat d'Histologia i Neurobiologia (UHNEUROB), c/Sant Llorenç 21, 43201 Reus, Spain.
Email: mariaangel.lanuza@urv.cat (M. A. L.) and josepmaria.tomas@urv.cat (J. M. T.)

Funding information
Ministerio de Economía y Competitividad (MINECO); Agencia Estatal de Investigación (AEI); European Regional Development Fund (ERDF), Grant/Award Number: SAF2015-67143-P and PID2019-106332GB-I00; Universitat Rovira i Virgili (URV), Grant/Award Number: 2017PFR-URV-B2-85; Catalan Government, Grant/Award Number: 2017SGR704; European Social Fund (ESF); Iniciativa de Empleo Juvenil (IEJ), Grant/Award Number: LE1511314-2014PEJ-04 and LE1911587-2019PEJ-04; Spanish Ministerio de Ciencia Innovación y Universidades, Grant/Award Number: PRE2020-092084 and PID2019-106332GB-I00; Universitat Rovira i Virgili (URV); Contractes de personal investigador predoctoral en formació, Grant/Award Number: 2018PMF-PIPF-18

Abstract

Neuromuscular junctions (NMJ) regulate cholinergic exocytosis through the M_1 and M_2 muscarinic acetylcholine autoreceptors (mAChR), involving the crosstalk between receptors and downstream pathways. Protein kinase C (PKC) regulates neurotransmission but how it associates with the mAChRs remains unknown. Here, we investigate whether mAChRs recruit the classical PKCβI and the novel PKCε isoforms and modulate their priming by PDK1, translocation and activity on neurosecretion targets. We show that each M_1 and M_2 mAChR activates the master kinase PDK1 and promotes a particular priming of the presynaptic PKCβI and ε isoforms. M_1 recruits both primed-PKCs to the membrane and promotes Munc18-1, SNAP-25, and MARCKS phosphorylation. In contrast, M_2 downregulates PKCε through a PKA-dependent pathway, which inhibits Munc18-1 synthesis and PKC phosphorylation. In summary, our results discover a co-dependent balance between muscarinic autoreceptors which orchestrates the presynaptic PKC and their action on ACh release SNARE-SM mechanism. Altogether, this molecular signaling explains previous functional studies at the NMJ and guide toward potential therapeutic targets.

KEYWORDS
muscarinic receptors, neuromuscular junction, PDK1, PKC, SNAP-25

Abbreviations: ACh, acetylcholine; Atr, atropine; Ca^{2+}, calcium; cPKC, classic PKC isoform; Dk, donkey; M_1, muscarinic acetylcholine receptor 1 subtype; M_2, muscarinic acetylcholine receptor 2 subtype; mAChR, muscarinic acetylcholine receptor; MARCKS, myristoylated alanine-rich C-kinase substrate; Met, methoctramine; Ms, mouse; Munc18-1, mammalian homologue of UNC-18; NMJ, neuromuscular junction; nPKC, novel PKC isoform; Pir, pirenzepine; PKC, protein kinase C; PKCβI, protein kinase C isoform Bi; PKCε, protein kinase C isoform ε; RACK1, receptor for activated C-kinase 1; Rb, rabbit; SNAP-25, synaptosome-associated protein 25; $βIV_{5-3}$, PKCβI inhibitor peptide; $εV_{1-2}$, PKCε inhibitor peptide.

N. Garcia, J. M. Tomàs, and M. A. Lanuza are equally contributed to this work.

1 | INTRODUCTION

Neuromuscular junctions (NMJ) express muscarinic acetylcholine receptors (mAChR) to sense how much acetylcholine is released and tune exocytosis in feedback. This coordination is achieved through the potentiating and inhibitory actions of M_1 and M_2 mAChR subtypes, respectively.[1] In general, the M_1 mAChR is classically associated to $G\alpha_q$ proteins and protein kinase C (PKC), whereas the M_2 mAChR is linked to $G\alpha_i$ proteins and protein kinase A (PKA) inhibition. However, this essential signaling is highly complex because it involves multiple downstream transduction pathways and the crosstalk between receptor subtypes. An example of this complexity at the NMJ is that the M_2 muscarinic receptor needs the association to M_1 to regulate the neuromuscular PKA molecular dynamics.[2] Also, the selective inhibition of both mAChR subtypes induce PKC action on NMJ neurotransmission[1] in which PKC plays an essential role.[3,4] At the adult NMJ, PKC coupling to ACh release requires a stimulus like calcium, presynaptic stimulation or the modulation of mAChR.[1,3,5] However, the molecular PKC signaling coupled to the ACh release associated to these signals, including the mAChR regulation, remains to be elucidated.

Previously to its activation, PKC undergoes a process of maturation (priming) to become competent to respond to second messengers.[6,7] PKC maturation involves three phosphorylation steps: the first is mediated by the phosphoinositide-dependent kinase 1 (PDK1) in the activation loop of the catalytic domain and the second and third are PKC autophosphorylations in the turn and hydrophobic motifs of the carboxy-terminal region.[8,9] Once matured, PKC stays in the cytosol in an inactive conformation ready to be activated.[9-11] Although PKC maturation was initially seen as constitutive, further studies found several stimuli which can induce it.[12-15] Finally, PKC activation requires its recruitment to the membrane, which is driven by binding to calcium, diacylglycerol and phosphatidylserine for classical PKC isoforms (cPKCs) and diacylglycerol and phosphatidylserine for novel PKC isoforms (nPKCs).[16] To end their signal, active PKCs are prone to be ubiquitinated and undergo activation-induced degradation.[17-20]

PKC phosphorylates many targets, which participate in general intracellular processes as well as neurotransmitter release. However, little is known about the role of each PKC isoform. Determining how extracellular receptors recruit specific PKC isoforms is crucial to predict how cells respond to extracellular signals. The cell components of the NMJ express different PKC isoforms, which likely help to finely regulate ACh release.[21-23] Of particular interest are PKCβI and PKCε, which are exclusive of the presynaptic nerve terminal and essential for ACh release.[22,24] For example, PKC phosphorylates the Ser^{306} and Ser^{313} of the accessory SNARE protein Munc18-1 (mammalian homologue of UNC-18), an essential, neuron-specific protein involved in neurotransmitter release[25-28] to prime vesicle fusion and increase the pool available for release.[29,30] At the NMJ, PKCβI and PKCε isoforms coordinately regulate Munc18-1 activity-dependent phosphorylation.[31] Another PKC substrate is SNAP-25 (synaptosome-associated protein 25), a component of the SNARE core complex. PKC phosphorylates SNAP-25 on Ser^{187}, a critical residue for calcium-triggered exocytosis.[28,32-36] This phosphorylation occurs after synaptic activity and high intracellular calcium and promotes vesicle pool refilling.[4,36,37] At the NMJ, PKCε regulates its activity-dependent phosphorylation.[38] Another example is MARCKS (myristoylated alanine-rich C-kinase substrate), a widely distributed PKC substrate which rearranges actin in the cytoskeleton in response to extracellular signals. Its phosphorylation is a marker of PKC activation in vivo[39] and it is also implicated in cholinergic neurosecretion and membrane trafficking.[40] At the NMJ, MARCKS phosphorylation has been linked with PKCε activity.[23]

Although PKC plays an essential role at the neurotransmission at the NMJ,[1] it is unknown the molecular PKC signaling coupled to the ACh release associated to its inducer signals, including the mAChR regulation. In the present work, we investigate whether M_1 and M_2 mAChRs regulate the PDK1-induced priming and recruit the classical PKCβI and the novel PKCε isoforms and modulate their maturation, membrane translocation and the phosphorylation of Munc18-1, MARCKS, and SNAP-25, crucial targets for neurosecretion. To analyze it, we studied the effect of muscarinic blockade on PDK1, PKCβI, and PKCε isoforms and their targets. Our results show a novel link between M_1 and M_2 signaling and the master kinase PDK1 and highlight the relevance of the balance between the presynaptic muscarinic autoreceptors M_1 and M_2 to influence a pool of PKC isoforms, which finely tuned the ACh release SNARE-SM mechanism.

2 | MATERIALS AND METHODS

2.1 | Animal welfare

The animals were cared for in accordance with the European Community Council Directive guidelines for the humane treatment of laboratory animals. Adult Sprague-Dawley rats (40-80 days; Criffa, Barcelona, Spain) were euthanized for tissue harvest and analysis. Animals were randomly assigned to the different treatments and at least three animals ($n \geq 3$) were used as biological replicates for every experiment detailed below. All animal work was approved by the Ethics Comitee of Animal Experimentation of the Universitat Rovira i Virgili.

2.2 | Chemicals

Muscarinic inhibition. Pirenzepine dihydrochloride (Tocris): 10 mM stock and used at 10 μM. Methoctramine tetrahydrochloride (Sigma): 1 mM stock and used at 1 μM. Atropine (Sigma): 200 μM stock and used at 2 μM.

PKC inhibition. The activity of PKC isoforms was blocked with PKC-derived peptides which compete for the receptor for activated C-kinase 1 (RACK1) binding and disrupt the cellular targeting of PKC isoforms. The PKCβI inhibitor βIV_{5-3} peptide[41,42] was kindly provided by Dr Mochly-Rosen from Stanford University and the PKCε inhibitor εV_{1-2} peptide[43] from MERCK. Both peptides are <40 amino acids (βIV_{5-3}, CKLFIMN; εV_{1-2}, EAVSLKPT). Briefly, blocking PKCβI with βIV_{5-3} did not affect PKCβII[44] and blocking PKCε with εV_{1-2} did not affect the novel PKCδ or classical PKC isoforms.[43,45,46] Furthermore, εV_{1-2} peptide has been validated with PKCε knockout mice.[47,48] Also, multiple sequence alignment reveals that βIV_{5-3} peptide shares 100% identity with PKCβI (Uniprot ID: P68403-1) and 0% identity with PKCε (Uniprot ID: P09216). Additionally, εV_{1-2} peptide shares 0% identity with PKCβI and 100% identity with PKCε. Working concentrations were optimized to 10 μM for βIV_{5-3}[22] and 100 μM for εV_{1-2}.[23] The difference in concentration was due to βIV_{5-3} peptide being connected to a deliverer peptide to enhance cell penetration.

PDK1 inhibition. PDK1 activity was blocked with GSK2334470, from MERCK. This highly specific inhibitor, only inhibited PDK1 activity without affecting 93 other kinases screened, including PKC and PKA.[49] GSK2334470 was made as 5 mM stock in DMSO and used at 2 μM on excised diaphragm muscles.

PKA inhibition. PKA activity was blocked with N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89, Calbiochem). H89 was made as 5 mM stock and used at 5 μM.

All chemicals were diluted in Ringer as specified and both control and drug-containing solutions contained dimethyl sulfoxide as vehicle at a final concentration of 0.1% (v/v).

2.3 | Treatments

The treatments were performed ex-vivo on excised diaphragm muscles. The experimental design was pairwise: one hemidiaphragm underwent the treatment while the other served as its paired control. In single-inhibitor treatments, the treated preparation was incubated for 60 minutes in Ringer solution containing the inhibitor. We used double-inhibitor treatments to study the implication of PKC in muscarinic signaling. In these, the treated hemidiaphragms were first preincubated for 30 minutes in Ringer solution containing a kinase inhibitor (βIV_{5-3}; εV_{1-2} or H89) and afterwards for further 30 minutes in Ringer solution containing the kinase inhibitor plus the muscarinic inhibitor indicated. The control pairs of the doubly inhibited preparations were incubated for 60 minutes in Ringer solution containing the appropriate kinase inhibitor to normalize its effects.

2.4 | Sample processing and fractionation

The diaphragm muscle was obtained from adult Sprague-Dawley rats (P40-60) immediately after euthanasia. Unconsciousness was induced by an intraperitoneal injection of tribromoethanol 2% (0.15 mL/10 g body weight) and afterwards euthanasia was performed through anaesthesic overdose. As indicated in the Directive 2010/63/EU and the Real Decreto 53/2013, death was confirmed by exsanguination. Diaphragms were excised with the phrenic nerve, taking special care to isolate the same nerve length and avoid connectivity damage.

Whole cell lysate. After treatment, muscles were immediately frozen in liquid nitrogen. Homogenization was performed with an overhead stirrer (VWR International, Clarksburg, MD) in ice-cold lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1% (Sigma, Saint Louis, MO, USA)). Insoluble materials were removed with two centrifugations at 4ºC: 1000g for 10 minutes and 15000g for 20 minutes. The final supernatant contained the whole cell fraction lysate.

Membrane/cytosol fractionated lysates. For membrane-cytosol fractionation, samples were immediately homogenized without freezing to avoid membrane damage before purification. The lysis buffer did not contain detergents (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; and protease inhibitor cocktail 1%). Insoluble materials were removed by centrifugation at 1000 *g* for 15 minutes at 4ºC. The resulting supernatant was further centrifuged at 130 000 g for 1 hour. The new supernatant corresponded to the cytosolic fraction while the pellet to the membrane fraction. The latter was resuspended in lysis buffer (in mM: NaCl 150, Tris-HCl (pH 7.4) 50, EDTA 1, NaF 50, PMSF 1, Na_3VO_4 1; NP-40 1%, Triton X-100 0.1%, and protease inhibitor cocktail 1%). The purity of the subcellular fractionation was determined with the cytosol-specific GAPDH and the membrane-specific Na^+/K^+-ATPase.

2.5 | Antibodies

The primary and secondary antibodies used are listed in Table 1. The anti-PKCε and anti-PKCβI antibodies were raised against their C-terminal region (human peptide), which share a low identity percentage (45%) between each

TABLE 1 Primary antibodies

Target	Immunogen	Origin	Company (ref)	Dilution
PDK1	Hu PDK1 residues 229-556	Ms mAb	Santa Cruz (sc-17765)	1/1000
pPDK1 (pSer^{241})	Hu PDK1 residues around pSer^{241}	Rb pAb	CST (3061)	1/1000
PKCβI	Hu PKCβI C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000
pPKCβI (pThr^{642})	Hu PKCβI residues 640-644	Rb pAb	Abcam (ab75657)	1/1000
PKCε	Hu PKCε C-terminus	Rb pAb	Santa Cruz (sc-214)	1/1000
pPKCε (pSer^{729})	Hu PKCε residues around pSer^{729}	Rb pAb	Santa Cruz (sc-12355)	1/1000
PKCα	Hu PKCα C-terminus	Rb pAb	Santa Cruz (sc-209)	1/1000
pPKCα (pSer^{657})	Phosphopeptide corresponding to the residues 654-663	Rb pAb	Upstate (06-822)	1/1000
Munc18-1	Hu Munc18-1 residues around Tyr^{157}	Rb mAb	CST (13414)	1/1000
pMunc18-1 (pSer^{313})	Hu Munc18-1 residues 307-319	Rb pAb	Abcam (ab138687)	1/1000
SNAP-25	Hu SNAP-25 residues around Gln^{116}	Rb mAb	CST (5309)	1/1000
pSNAP-25 (pSer^{187})	Rat SNAP-25 residues around pSer^{187}	Rb pAb	Abcam (ab169871)	1/1000
MARCKS	Hu MARCKS residues 2-66	Ms mAb	Santa Cruz (sc-100777)	1/1000
pMARCKS (pSer$^{152/156}$)	Rat MARCKS residues around pSer$^{152/156}$	Rb pAb	Sigma (07-1238)	1/1000
GAPDH	Rb GAPDH	Ms mAb	Santa Cruz (sc-32233)	1/2000
ATPase	Chicken ATPase residues 27-55	Ms mAb	DSHB (a6f)	1/2000
Secondary antibodies	Anti-Rb conjugated HRP	Dk pAb	711-035-152	1/10000
	Anti-Ms conjugated HRP	Rb pAb	A9044	1/10000
	Anti-goat conjugated Alexa fluor 568	Dk pAb	A-11057	1/500

Note: Antibodies used in this study and procedure specifications.

Abbreviations: Dk, donkey; Hu, human; mAb, monoclonal antibody; Ms, mouse; pAb, polyclonal antibody; Rb, rabbit.

other. These antibodies were validated in Hurtado et al and Simó et al.[22,31] In brief, the incubation with the $\varepsilon V_{1\text{-}2}$ peptide for 30 minutes decreases PKCε and pPKCε levels and the incubation with the $\beta IV_{5\text{-}3}$ peptide decreases PKCβI and pPKCβI levels. The anti-Munc18-1 antibody epitope, the residues surrounding Tyr^{157}, is not conserved in other Munc18 isoforms, and the anti-pMunc18-1 Ser^{313} antibody was raised against a synthetic peptide corresponding to the

human Munc18-1 region 307-319, around the PKC target. The anti-SNAP-25 antibody was raised against the human peptide surrounding residues of Gln^{116}, which are not conserved in other SNAP family members (identity percentages in rat: SNAP-25 100%; SNAP-23 63%; SNAP-47 25%; SNAP-29 13%). SNAP-25 antibody showed the typical pattern of tissue expression previously known, different from SNAP-23,[50] making cross-reactivity less likely. On the other hand, phosphorylated SNAP-25 (pSNAP-25) at Ser^{187}, was detected with an antibody raised against the residues 182-192 of the protein. This sequence differs from SNAP-23 (identity percentage in rat: 73%); SNAP-47 (9%), and SNAP-29 (27%). Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw).[51] Moreover, the phosphospecificity was proven by the absence of signal after incubation with the antigen phosphopeptide[52] and after treatment with lambda phosphatase (manufacturer's datasheet).

As a control for western blot, when primary antibodies were omitted, the membranes never revealed staining due to the secondary antibody. Pretreatment of a primary antibody with the appropriate blocking peptide (between three- and eightfold by weight) in skeletal muscle tissue prevented immunolabeling. The incubation with the specific $\varepsilon V_{1\text{-}2}$ peptide for 30 minutes decreases PKCε and pPKCε levels, whereas the incubation with the $\beta IV_{5\text{-}3}$ peptide decreases PKCβI and pPKCβI levels.[31] As a control for immunohistochemistry, several muscles were incubated omitting the primary antibody, which always abolished any positive staining.

2.6 | Western blotting

Sample protein content was determined with the DC protein assay (Bio-Rad, CA, USA). Volumes containing 30 μg of protein were separated at 110 V through 8% SDS-PAGE gels (10% to detect SNAP-25) and electrotransferred to PVDF membranes (Bio-Rad, CA, USA). Blocking solutions were tris-buffered saline with Tween-20 containing 5% nonfat dry milk or 5% BSA. Primary antibodies were incubated overnight at 4°C and HRP-conjugated secondary antibodies for 1 hour. Chemiluminescence was revealed with an ECL kit (GE Healthcare Life Sciences, UK) and imagined with the ChemiDoc XRS+Imaging System (Bio-Rad, CA, USA).

The optical density of the bands was calculated with ImageJ software, always from the same immunoblot image. The values were normalized to the background value and to the total protein transferred, analyzed with SYPRO Ruby protein blot stain, (Bio-Rad, CA, USA). Foldchanges between treatment and control were always calculated from the same immunoblot image. All presented data derive from densitometry measurements made of 3-10 separate replicates, plotted against controls.

2.7 | Immunohistochemistry

The NMJ of diaphragm and *levator auris longus* (LAL) muscles were analyzed by immunohistochemistry with identical outcomes. The thinness of LAL muscles improves image quality and analysis of NMJs. Whole muscle mounts were fixed with 4% paraformaldehyde for 30 minutes. After fixation, the muscles were rinsed with PBS and incubated in 0.1 M glycine in PBS. The following incubations were performed overnight at 40°C: first, permeabilization with 1% Triton X-100 in PBS. Then, blocking of nonspecific binding with 4% BSA in PBS. Next, muscles were incubated also overnight with the appropriate primary antibody. After five washing steps, the muscles were incubated at 4°C with the appropriate secondary antibody together with α-bungarotoxin (α-BTX) conjugated with TRITC, to detect nicotinic acetylcholine receptors (nAChRs). Immunolabeled NMJs from the whole-mount muscles were viewed with a laser-scanning confocal microscope (Nikon TE2000-E). Special consideration was given to the possible contamination of one channel by another. In experiments involving negative controls, the photomultiplier tube gains and black levels were identical to those used for a labeled preparation made in parallel with the control preparations. Images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) and neither the contrast nor brightness were modified.

2.8 | Statistics

The ratio between the experimental and control samples was calculated densitometry measures of the same image Values are presented as mean ± standard deviation (SD). Shapiro-Wilk test was used to test sample normality. Statistical difference was determined with paired Student's *t* test or its non-parametric alternative Wilcoxon test. Multiple comparisons were corrected with the Holm-Sidak method (GraphPad Prism, San Diego, USA). The significance threshold was $*P < .05$, $**P < .01$, and $***P < .001$.

3 | RESULTS

3.1 | M_1 associates to PKCβI and PKCε and M_2 to PKCε

First, we studied how M_1 and M_2 mAChR affect PKCβI and PKCε isoforms at the NMJ. We used antibodies with high specificity for the corresponding protein at the predicted molecular weight: pPKCβI (Thr^{641}) 77 kDa, PKCβI 79 kDa, pPKCε (Ser^{729}) 90 kDa, PKCε 82 kDa (Figure 1A). The anti-pPKCβI antibody detected a second band corresponding to the PKC catalytic domain. This band was not affected by

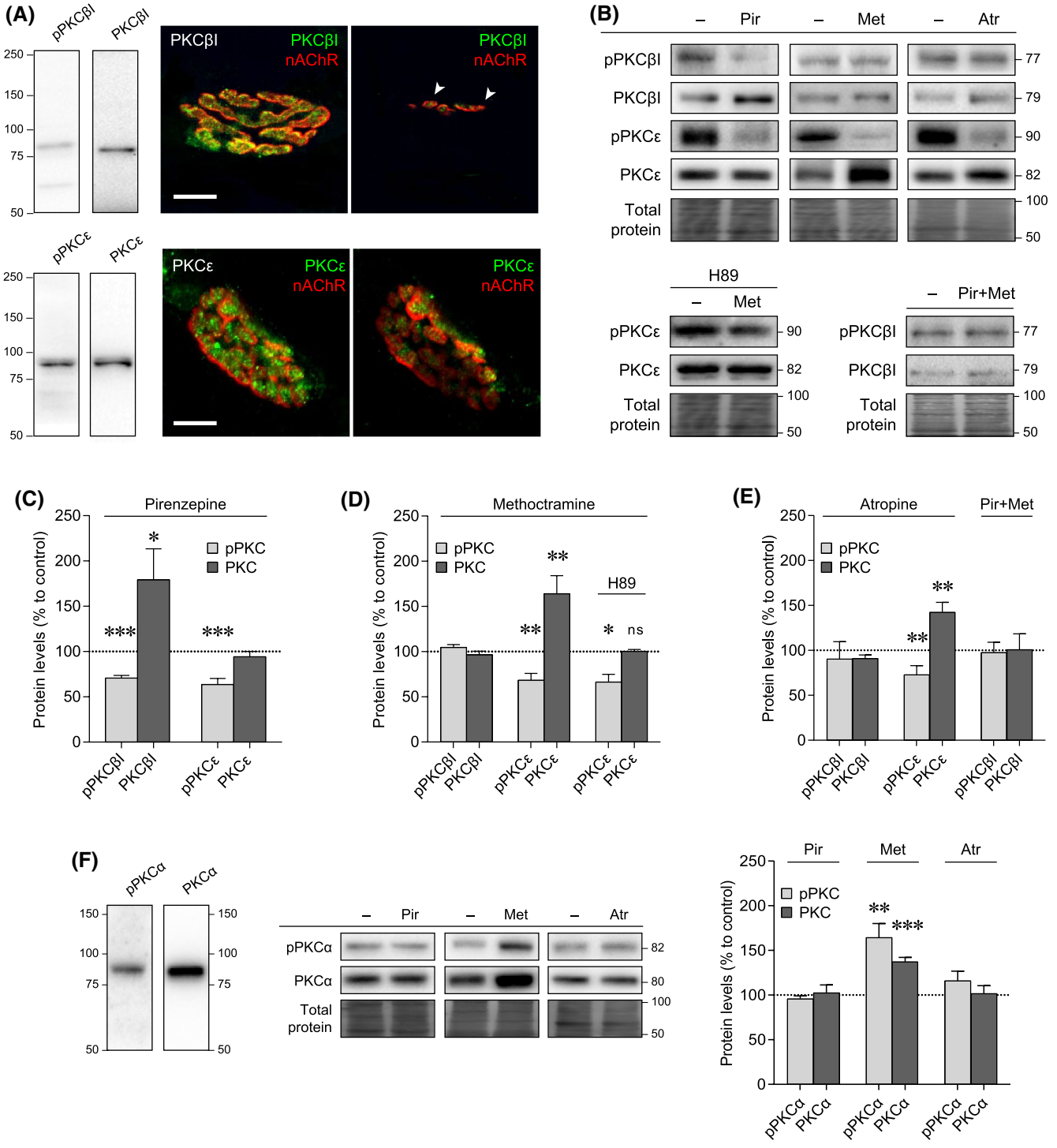

FIGURE 1 PKCβI, PKCε, and PKCα levels and maturation after muscarinic blockade. A, Molecular weight of the phosphorylated and total PKC βI isoform (pPKCβI and PKCβI) and PKC ε isoform (pPKCε and PKCε) at the rat diaphragm. Immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and PKCβI in green or PKCε in green. Scale bars = 10 μm. B, Western blot bands of PKC isoforms. C, Effect of M_1 inhibition (pirenzepine, Pir). D, Effect of M_2 inhibition (methoctramine, Met) and M_2 inhibition after a pretreatment with the PKA blocker H89. E, Effect of M_1/M_2 inhibition with atropine (Atr) and with a mixture of pirenzepine and methoctramine (Pir+Met). F, Molecular weight of the phosphorylated and total PKC α isoform (pPKCα and PKCα) at the rat diaphragm and effect of the muscarinic inhibitors. M_1 promotes the priming and activation of PKCβI whereas M_2 promotes PKCε. H89: PKA blocker. Data are expressed as percentage of protein levels after treatment. Mean value ± SD. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

the treatments and in 8% SDS-PAGE gels it could be well-distinguished from the predicted 79 kDa full-length pPKCβI band. The PKCβI and PKCε are exclusively presynaptic at the NMJ[22,23] and here we also show that their immunohistochemical staining is limited to the presynaptic region over the nicotinic acetylcholine receptors (nAChRs) (see arrows in the confocal section image of PKCβI) and does not surpass areas outside the NMJ endplate. The first two indicators of PKC activity that we studied were PKC phosphorylation, which indicates PKC priming, and protein level, which indicates PKC synthesis or activity-induced degradation (Figure 1B). PKC turnover is activity-dependent and can be used as marker of its activity.[17-20]

M_1 blockade with pirenzepine (Pir) decreased PKCβI Thr^{641} phosphorylation and increased its protein amount (Figure 1C). This increase in PKCβI levels along with the decrease in PKCβI priming could indicate an accumulation of the inactive kinase. Additionally, M_1 blockade decreased PKCε Ser^{729} phosphorylation without affecting its protein amount. Altogether, this suggests that M_1 signaling promotes the priming of both PKC βI and ε isoforms and probably reduces PKCβI levels due to activity-dependent degradation at basal conditions.

M_2 blockade with methoctramine (Met) did not affect PKCβI phosphorylation or its total levels (Figure 1D). Surprisingly, M_2 inhibition decreased the phosphorylation of PKCε and upregulated its total amount. Because the action of M_2 on pPKCε/PKCε is similar to that of M_1 on pPKCβI/PKCβI (Figure 1C-D), M_2 could be inducing the priming of PKCε and decreasing its protein level. To understand if M_2 acts directly on PKCε or it involves the classic mediator PKA, we used the inhibitor H89, a blocker of PKA catalytic subunits. A previous incubation with H89 before methoctramine abolished the increase in PKCε protein levels without altering the decrease in PKCε phosphorylation (Figure 1D). Altogether, these results show that M_2 signaling modulates PKCε rather than PKCβI, involving PKA to decrease PKCε levels and promoting PKCε phosphorylation through a mechanism that does not require PKA.

The combined action of M_1 and M_2 mAChR pathways can be determined with atropine (Atr), a well-known muscarinic pan-inhibitor. In particular, atropine has the same affinity for M_1 and M_2 and its effect on ACh release and PKA signaling is mimicked by a mixture of Pir +Met at NMJs.[2,53] The comparison between the effects of subtype-selective inhibitors versus atropine reveals which subtype has higher overall impact on each PKC isoform. Figure 1E shows that M_1/M_2 blockade with Atr did not affect PKCβI but increased the total levels of PKCε and decreased its phosphorylation. Compared to the results of the selective inhibitions, both M_1 and M_2 inhibitions are responsible for the downregulation of PKCε priming, whereas M_2 inhibition is responsible for the increase in PKCε protein levels. Interestingly, atropine does not replicate the effects of pirenzepine on PKCβI. This might suggest that M_1 inhibition needs an active M_2 to prime PKC βI and decrease its levels. In addition to Atr, we used a mixture of pirenzepine and methoctramine (Pir+Met) to check the implication of both M_1 and M_2 mAChRs over PKCβI. The Pir+Met treatment minimizes the participation of other mAChR subtypes (Figure 1E). Therefore, it is useful to check if the effect of atropine can be explained by mainly M_1 and M_2 blockade or, otherwise involves other mAChR subtypes. We found that Pir+Met incubation did not modify PKCβI protein levels and phosphorylation, mimicking atropine's effect. This reinforces that M_1 mAChR needs an active M_2 to modify PKCβI levels.

To check the scope of muscarinic signaling, we studied how muscarinic inhibition affects the PKCα isoform, which is mostly expressed at the post-synaptic muscle.[5,54,55] Unlike the presynaptic PKCβI and PKCε, pirenzepine and atropine did not affect PKCα (Figure 1F). However, M_2-blockade increased both PKCα protein levels and phosphorylation. This result indicates that M_2 signaling reduces PKCα priming and protein amount.

In summary, M_1 promotes the priming of PKCβI and PKCε and downregulates PKCβI probably through activation-induced degradation, which needs an active M_2. On the other hand, M_2 promotes PKCε priming and decreases its protein levels through PKA.

3.2 | M_1 translocates PKCβI and PKCε whereas M_2 only PKCε

Next, we studied how muscarinic signaling modulates the subcellular location of PKC isoforms in the membrane, which is a surrogate measure of PKC isoform activation.[56] To test the action of M_1 and M_2, we separated the membrane (particulate) and cytosolic (soluble) fractions of the samples after treatment. The membrane fraction contains detergent-insoluble compartments, including the plasma membrane, intracellular vesicles and other intracellular membranous compartments. We checked the fractionation purity with the cytosol marker GAPDH and the membrane marker Na^+/K^+-ATPase, which were highly enriched in their corresponding fraction and essentially undetectable on the opposite.

Figure 2 shows the distribution of the PKC isoforms between the membrane and cytosol. We set the value of control samples as 100% (membrane + cytosol) and calculated the treatment values in relation to the control. PKCβI and PKCε isoforms were more associated to the membrane regardless of their phosphorylation (proportion membrane–cytosol from control samples: pPKCβI 79-21% ± 4; PKCβI 79-21% ± 3; pPKCε 81-19% ± 5; PKCε 68-32% ± 5).

The effect of M_1 inhibition with Pir on PKCβI was limited to the membrane, decreasing its phosphorylation and

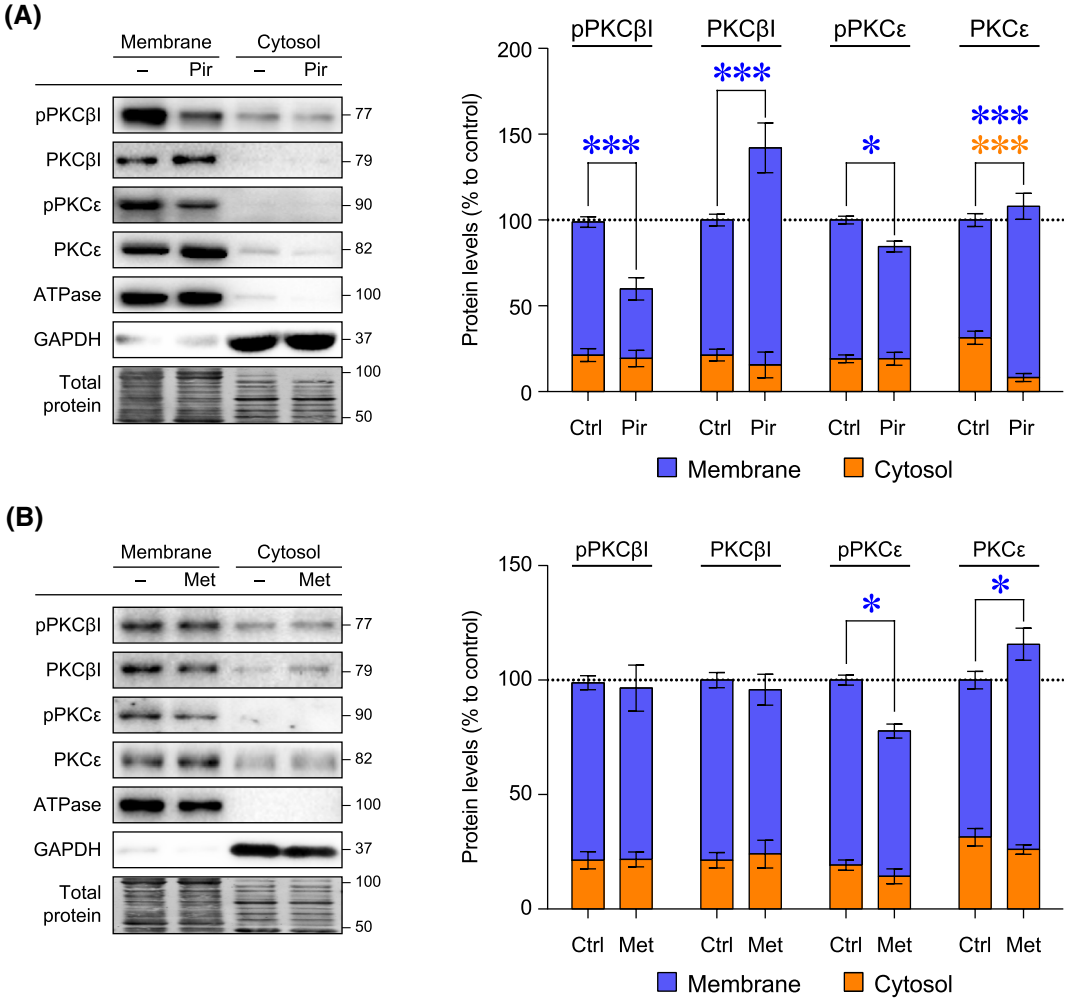

FIGURE 2 Distribution of the PKC isoforms between the membrane (particulated) and cytosol (soluble) fractions after muscarinic blockade. A, Western blot bands of PKC isoforms and effect of M_1 inhibition (pirenzepine, Pir). B, Western blot bands of PKC isoforms and effect of M_2 inhibition (methoctramine, Met). PKCβI membrane association is orchestrated by M_1 whereas that of PKCε is modulated by M_1 and M_2. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). Control value (Ctrl) is set at 100% (membrane + cytosol) and the treatment value is calculated in relation to the control. $^*P < .05$, $^{***}P < .001$

increasing the total protein levels (Figure 2A). The small amount of cytosolic PKCβI both before and after pirenzepine treatment makes translocation unlikely to explain the great increase in the membrane. Therefore, after M_1 blockade, PKCβI is accumulated on the membrane. Regarding PKCε, M_1 blockade decreased the phosphorylated PKCε in the membrane, accumulated total PKCε in the membrane and decreased it from the cytosol (see discussion) These results indicate that M_1 inhibition downregulates PKCε phosphorylation and induces its accumulation at the membrane.

M_2 inhibition with Met (Figure 2B) did not affect the subcellular distribution of pPKCβI or PKCβI, reinforcing the pervious conclusion that M_2 signaling does not involve PKCβI. Regarding PKCε, M_2 inhibition downregulated PKCε phosphorylation and increased its protein levels in the membrane fraction. In summary, M_2 blockade seems to inhibit the priming of PKCε and accumulate PKCε levels on the membrane.

3.3 | Both M_1 and M_2 activate PDK1

After observing that pirenzepine decreased the phosphorylation of PKCβI and PKCε and methoctramine decreased the phosphorylation of PKCε, we wondered if that was caused by an effect of muscarinic signaling over the priming of PKC. Thus, we studied the PKC-priming kinase PDK1 and its phosphorylation on Ser^{241} after M_1 and M_2 blockade. The antibodies detected a band of 60 kDa for both pPDK1 and PDK1 (Figure 3A). PDK1 is a synaptic kinase[15] and here we demonstrate that it is localized in the presynaptic region over postsynaptic nAChRs gutters (see the confocal section image at the right) and does not surpass areas outside the NMJ endplate.

The phosphorylation of PDK1 decreased after both pirenzepine and methoctramine treatments without affecting PDK1 protein levels (Figure 3B). The treatment with the pan-inhibitor atropine also decreased PDK1 phosphorylation without

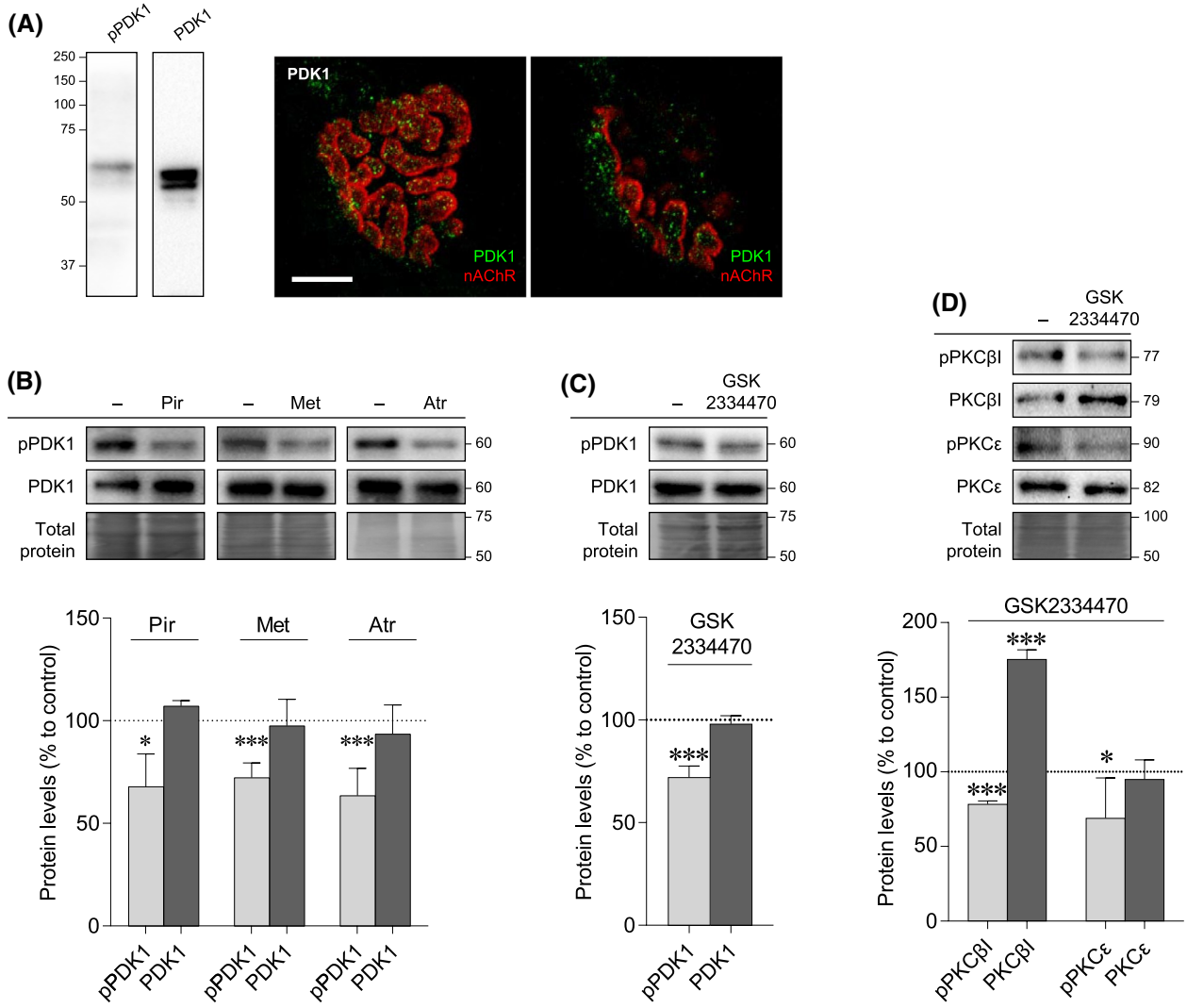

FIGURE 3 PDK1 protein levels and phosphorylation after muscarinic blockade. A, Molecular weight of the phosphorylated and total PDK1 (pPDK1 and PDK1) at the rat diaphragm. At the right: immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and PDK1 in green. Scale bars =10 μm. B, Effect of M_1 inhibition (pirenzepine, Pir), M_2 inhibition (methoctramine, Met) and M_1/M_2 inhibition (atropine, Atr). Both M_1 and M_2 pathways promote the phosphorylation of PDK1. C, Effect of PDK1 inhibition (GSK2334470) over the phosphorylated and total PDK1. D, Effect of PDK1 inhibition over the phosphorylated and total PKCβI and PKCε isoforms. Data are expressed as percentage of protein levels after treatment. Mean value ± SD. $^*P < .05$, $^{***}P < .001$

affecting PDK1 protein levels. This decrease in PDK1 phosphorylation correlates with the accompanying decrease in phospho-PKC previously detected (note that M_2 only affects PKCε; Figure 1D). To understand whether the signaling of both M_1 and M_2 mAChRs might be promoting PKC priming through PDK1, we studied the effect of PDK1 blockade. The specific PDK1 inhibitor GSK2334470 decreased the phosphorylation of PDK1 Ser^{241} without changing its protein levels (Figure 3C). Next, we tested whether PDK1 inhibition affects the downstream PKC isoforms. PDK1 inhibition decreased PKCβI phosphorylation and increased its protein levels (Figure 3D). PDK1 inhibition per se seems to reproduce the effect that M_1 inhibition with pirenzepine has over PKCβI. Regarding the PKCε isoform, PDK1 inhibition decreased its phosphorylation.

3.4 | M_1 and M_2 mAChR subtypes control PKC substrates

Because M_1 and M_2 mAChRs recruit specific PKC isoforms, they likely influence PKC substrates differently. Thus, we studied how M_1 and M_2 blockade affected the PKC targets Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}) and MARCKS ($Ser^{152/156}$) (Figure 4A-B).

M_1 inhibition downregulated the phosphorylation of all PKC substrates: Munc18-1 phospho-Ser^{313}, SNAP-25 phospho-Ser^{187} and MARCKS phospho-$Ser^{152/156}$ without affecting their protein amount (Figure 4C). Along with the decrease in the PKC-priming kinase PDK1, this result supports the interpretation that the changes observed over PKCβI and

PKCε after M_1 inhibition correspond to a decrease in PKC activity.

In opposition of M_1 inhibition, M_2 blockade increased the phosphorylation of some PKC substrates. This is the case of Munc18-1, whose phosphorylation and protein levels were increased by Met (Figure 4D). This suggests that M_2 signaling downregulates Munc18-1 synthesis, which possibly affects its phosphorylation levels. On the other hand, M_2 inhibits MARCKS phosphorylation, which is shown by M_2 inhibition upregulating MARCKS phosphorylation without changing its protein levels. In contrast, M_2 inhibition did not affect SNAP-25 Ser^{187} phosphorylation or its total protein levels, being the only substrate not affected by M_2 signaling.

Once determined the role of M_1 and M_2 subtypes per se, we proceeded to study their combined action on PKC substrates (Figure 4E). The M_1/M_2 inhibition with Atr decreased

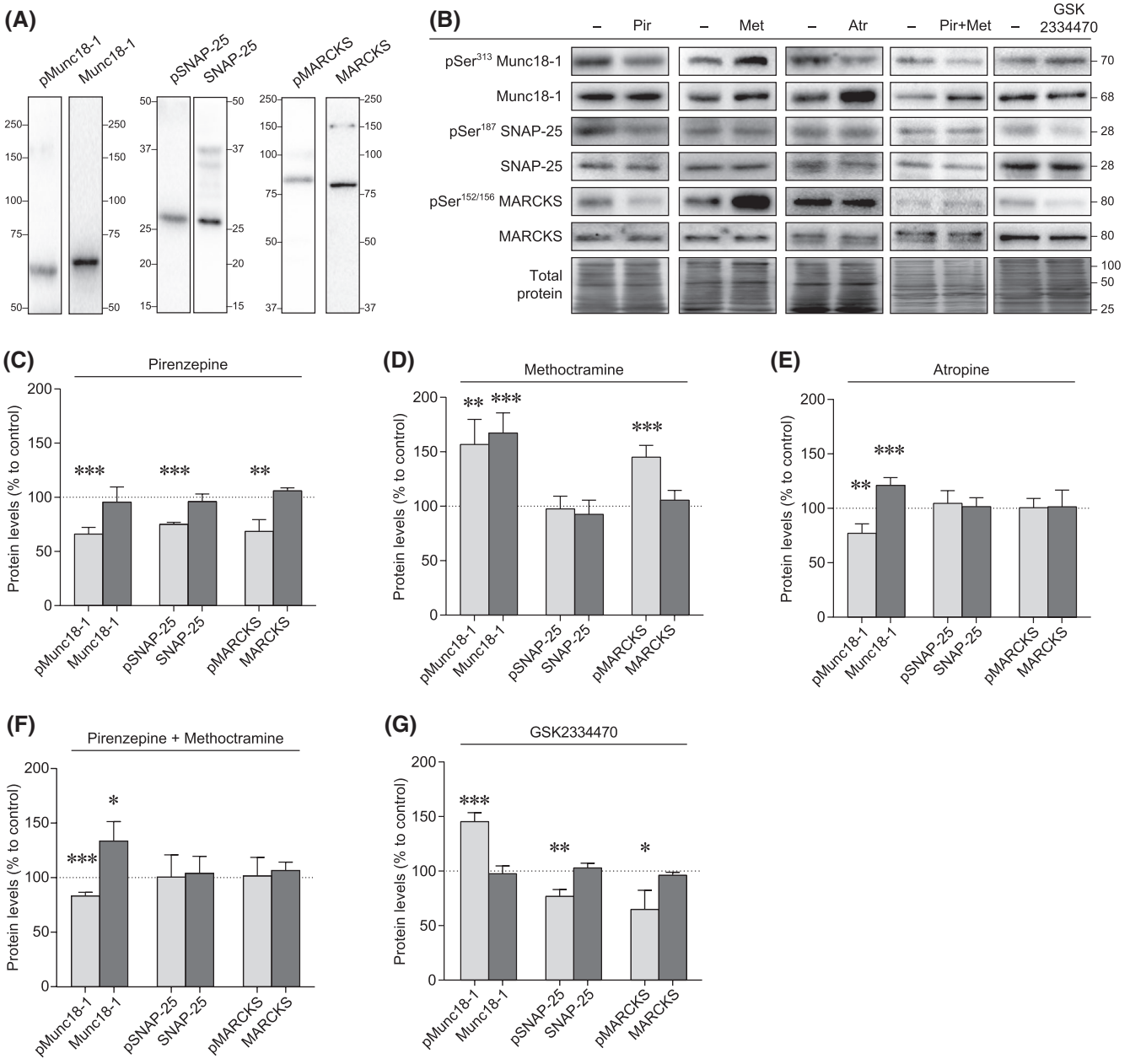

FIGURE 4 Munc18-1, SNAP-25, and MARCKS phosphorylation and protein levels after muscarinic blockade. A, Molecular weight of the phosphorylated and total Munc18-1 (pMunc18-1 and Munc18-1), SNAP-25 (pSNAP-25 and SNAP-25), and MARCKS (pMARCKS and MARCKS) at the rat diaphragm. B, Western blot bands of Munc18-1, SNAP-25 and MARCKS. C, Effect of M_1 inhibition (pirenzepine, Pir). D, Effect of M_2 inhibition (methoctramine, Met). E, Effect of M_1/M_2 inhibition (atropine, Atr). F, Effect of M_1/M_2 inhibition with a Pir and Met mixture (Pir+Met). G, Effect of PDK1 inhibition (GSK2334470). M_1 promotes the phosphorylation of all PKC substrates Munc18-1, SNAP-25, and MARCKS whereas the M_2 inhibits Munc18-1 levels and MARCKS phosphorylation. Both receptors converge on PDK1, whose activity promotes the phosphorylation of SNAP-25 and MARCKS and inhibits that of Munc18-1. Data are expressed as percentage of protein levels after treatment. Mean value ±SD. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

the phosphorylation of Munc18-1 and increased the total levels of Munc18-1. After comparing with the subtype selective inhibitors, M_1 inhibition seems responsible for the decrease in pMunc18-1, while the increase in Munc18-1 seems caused by M_2 inhibition. The M_1/M_2 inhibition with atropine did not change MARCKS phosphorylation or protein levels. This outcome on pMARCKS is in line with the action of Pir and Met and is possibly the result of the balance between the M_1-increasing and M_2-decreasing actions. Moreover, SNAP-25 $pSer^{187}$ remained unaltered after M_1/M_2 blockade. Interestingly, atropine does not replicate the effect of pirenzepine, which may indicate that M_1 needs M_2 active to promote SNAP-25 phosphorylation. Alternatively, atropine could affect other receptors like M_4 mAChR which could counter the action of M_1. To rule out the effect of atropine on other muscarinic receptors, we tested the effect of a mixture of pirenzepine and methoctramine (Pir + Met) (Figure 4F). An incubation with Pir + Met replicated the same results as atropine over Munc18-1, SNAP-25 and MARCKS, reinforcing the participation of solely the M_1 and M_2 mAChR subtypes.

Finally, because we determined that M_1 and M_2 selective blockers downregulate PDK1 activity at the NMJ, we analyzed whether the inhibition of PDK1 per se could also affect the downstream PKC substrates (Figure 4G). PDK1 inhibition with GSK2334470 increased the phosphorylation of Munc18-1 without affecting its protein amount. On the other hand, PDK1 inhibition decreased the phosphorylation of SNAP-25 $pSer^{187}$ and MARCKS $pSer^{152/156}$ without affecting their total levels. These data show that PDK1 blockade replicates the effect of M_2 inhibition over Munc18-1 and the effect of M_1 inhibition over SNAP-25 and MARCKS, reinforcing that PDK1 is at the crossroad between M_1 and M_2 signaling over PKC.

3.5 | M_1 signaling involves PKCβI and PKCε to phosphorylate Munc18-1, MARCKS, and SNAP-25

To determine the M_1 and M_2 mAChR pathways more accurately, we investigated the involvement of PKC isoforms by studying if the specific competitive peptides $\beta IV_{5\text{-}3}$ (inhibitor of PKCβI) and $\varepsilon V_{1\text{-}2}$ (inhibitor of PKCε) could prevent the effects of the selective muscarinic blockades. Also, because we detected that PKA was involved in the regulation of M_2 on PKC and some PKC substrates have PKA phosphorylation sites (see discussion), we also used H89 to determine the PKA role.

A previous incubation with $\beta IV_{5\text{-}3}$ or $\varepsilon V_{1\text{-}2}$ before pirenzepine (Figure 5A) abolished the effect of M_1 blockade on Munc18-1 phosphorylation (Figure 5B). This indicates that both PKCβI and PKCε are necessary for M_1 mAChR action on Munc18-1 at the NMJ. In contrast, H89 did not prevent pirenzepine from reducing Munc18-1 phosphorylation, which indicates that PKA does not participate in M_1/Munc18-1 phosphorylation.

All previous inhibition of PKCβI, PKCε or PKA prevented pirenzepine from reducing SNAP-25 phosphorylation (Figure 5C). This indicates that M_1 mAChR action on SNAP-25 needs both PKC and PKA kinases, suggesting that it is more controlled than Munc18-1 phosphorylation.

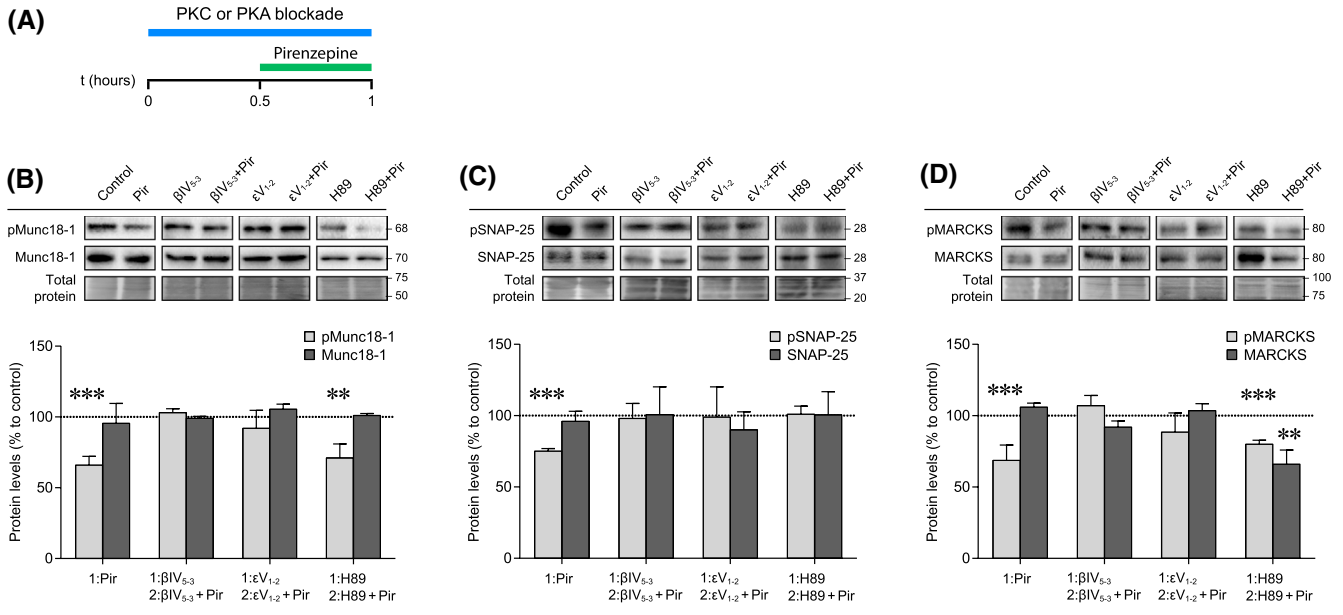

FIGURE 5 Effect of PKC and PKA inhibitors on the action of M_1 over PKC substrates. A, The specific inhibitors of PKCβI ($\beta V_{5\text{-}3}$), PKCε ($\varepsilon V_{1\text{-}2}$), and PKA (H89) were pre-incubated before M_1 blockade (pirenzepine, Pir) to determine the requirement of each kinase. B, Western blot bands and optical densitometry of phosphorylated (pMunc18-1) and total Munc18-1. C, Western blot bands and optical densitometry of phosphorylated (pSNAP-25) and total SNAP-25. D, Western blot bands and optical densitometry of phosphorylated (pMARCKS) and total MARCKS. Data are mean values ±SD. $^{**}P < .01$, $^{***}P < .001$

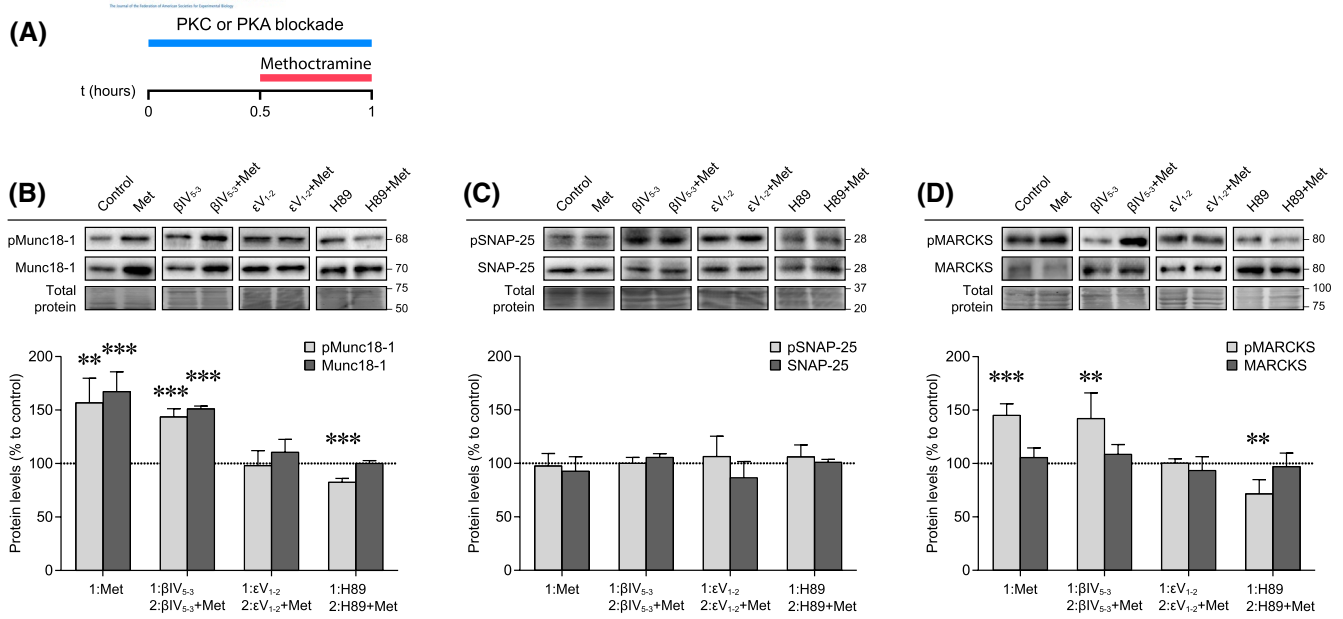

FIGURE 6 Effect of PKC and PKA inhibitors on the action of M_2 over PKC substrates. A, The specific inhibitors of PKCβI ($\beta V_{5\text{-}3}$), PKCε ($\varepsilon V_{1\text{-}2}$), and PKA (H89) were pre-incubated before M_2 blockade (methoctramine, Met) to determine the requirement of each kinase. B, Western blot bands and optical densitometry of phosphorylated (pMunc18-1) and total Munc18-1. C, Western blot bands and optical densitometry of phosphorylated (pSNAP-25) and total SNAP-25. D, Western blot bands and optical densitometry of phosphorylated (pMARCKS) and total MARCKS. Data are mean values ±SD. $^{**}P < .01$, $^{***}P < .001$

Finally, the previous incubation with $\beta IV_{5\text{-}3}$ abolished the downregulation of pirenzepine on MARCKS phosphorylation without modifying MARCKS protein levels (Figure 5D). A previous blockade of PKCε also prevented pirenzepine from affecting MARCKS phosphorylation and protein levels. This indicates that M_1 mAChR action on MARCKS needs both PKCβI and PKCε. In contrast, deleting PKA activity with H89 did not prevent pirenzepine from reducing MARCKS phosphorylation. Surprisingly, we found a decrease in MARCKS levels. Because PKA is involved in protein translation (see discussion), shutting down PKA from the system might be revealing a MARCKS-degrading pathway induced by M_1 mAChR.

3.6 | M_2 signaling involves PKCε and PKA to reduce Munc18-1 level and MARCKS phosphorylation

We previously found that M_2 blockade upregulated Munc18-1 levels and phosphorylation, probably by enhancing Munc18 synthesis and, hence, its phosphorylation levels. The PKCβI isoform is dispensible for this mechanism, because a previous incubation of the PKCβI inhibitor $\beta IV_{5\text{-}3}$ did not prevent the effect of methoctramine (Figure 6A-B). Instead, Munc18-1 upregulation was abolished by a previous inhibition of PKCε ($\varepsilon V_{1\text{-}2}$) and PKA (H89). This indicates that M_2 mAChR requires PKCε and PKA, but not PKCβI, to decrease Munc18-1 at the NMJ. In line with the previous results, where M_2 inhibition did not affect SNAP-25, none of the PKC and PKA inhibitors added any effect on the methoctramine treatment (Figure 6C).

We also studied the PKC and PKA role on the upregulation of phospho-MARCKS after M_2 inhibition (Figure 6D). Previously blocking PKCβI did not prevent M_2 modulation, because Met still increased MARCKS phosphorylation. However, a previous inhibition of PKCε ($\varepsilon V_{1\text{-}2}$) and PKA (H89) abolished the upregulation of phospho-MARCKS after M_2 inhibition. This indicates that M_2 mAChR requires PKCε and PKA, but not PKCβI, to decrease pMARCKS at the NMJ. Interestingly, without PKA, M_2 blockade decreased Munc18-1 and MARCKS phosphorylation, which is coincident with the decrease in phospho-PKCε on the same conditions (Figure 1D).

3.7 | Muscarinic subtypes induce the translocation of Munc18-1, SNAP-25, and MARCKS

We investigated the distribution between the membrane and cytosol of Munc18-1, MARCKS and SNAP-25 after muscarinic inhibition to clarify the implications of the previous regulations (Figure 7). At basal conditions, Munc18-1 was strongly associated to the membrane, both in its phosphorylated form pMunc18-1 (membrane–cytosol, 85-15% ± 4) as well as its total levels (76-24% ± 3). Similarly, pSNAP-25 Ser^{187} was also associated to the membrane (77-23% ± 3) as well as total SNAP-25

(77-23% ± 4). Phosphorylated MARCKS was equally distributed between the two fractions (55-45% ± 5) and MARCKS slightly more to the cytosol (38-62%±6).

The decrease in Munc18 phosphorylation after M_1 inhibition was located at the membrane fraction (Figure 7A). This reduction in pMunc18-1 was accompanied by a translocation of the total Munc18-1 species from the membrane to the cytosol. The decrease in SNAP-25 phosphorylation after M_1 inhibition occurred at the membrane fraction. However, this decrease did not affect the distribution of the total SNAP-25. Similar to SNAP-25, M_1 inhibition also decreased MARCKS phosphorylation on the membrane compartment without affecting the distribution of the total MARCKS. Altogether, these results indicate that M_1 signaling promotes Munc18 (Ser^{187}) phosphorylation and its association to the membrane. M_1 also induces the phosphorylation of SNAP-25 and MARCKS on the membrane without affecting their membrane translocation.

M_2 inhibition with Met increased the phosphorylation of Munc18-1 in the membrane without affecting it on the cytosol (Figure 7B). Additionally, M_2 inhibition also upregulated the total protein Munc18-1 levels in the membrane fraction rather than in the cytosol. In consistency with the previous results, the blockade of M_2 did not affect SNAP-25 Ser^{187} phosphorylation and it did not induce the translocation of its protein levels. Finally, the increase in MARCKS phosphorylation by Met was concentrated in the membrane fraction without affecting the phosphorylation on the cytosol. This M_2 modulation did not affect the total levels of MARCKS, which remained unchanged in both the membrane fraction and in the cytosol.

3.8 | The synaptic region contains the M_2 signaling on PKCβI, PKCε, Munc18-1, and SNAP-25

To study the muscarinic signaling that occurs at the presynaptic terminal, we selected PKC isoforms that participate in neurotransmitter release and are exclusively expressed at the presynaptic terminal of the NMJ.[5,22,24] However, it is not possible to isolate the phrenic nerve and preserve its

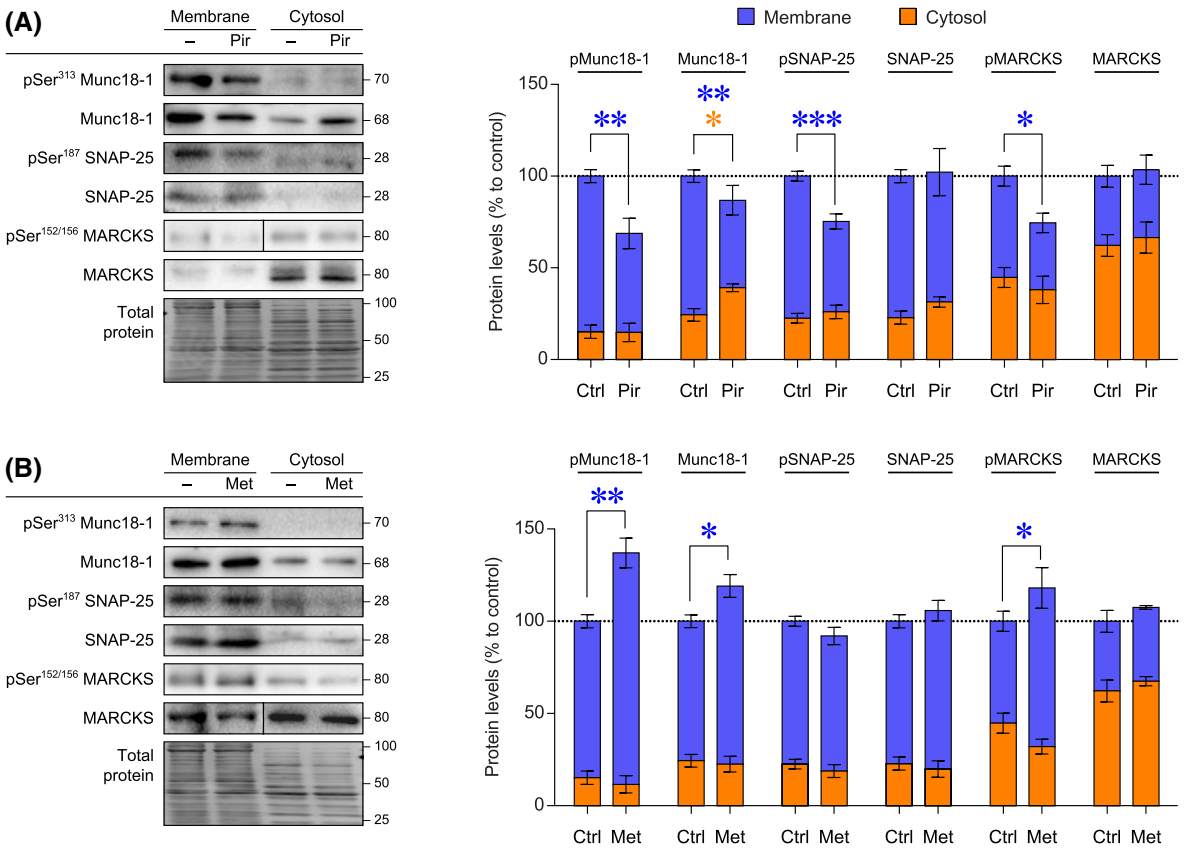

FIGURE 7 Distribution of the PKC substrates between the membrane (particulated) and cytosol (soluble) fractions after muscarinic blockade. A, Western blot bands and optical densitometry of PKC substrates after M_1 blockade (pirenzepine, Pir). B, Western blot bands and optical densitometry of PKC substrates after M_2 blockade (methoctramine, Met). M_1 promotes Munc18 phosphorylation and translocation to the membrane. Additionally, M_1 induces SNAP-25 and MARCKS phosphorylation on the membrane without affecting their translocation. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). Control value (Ctrl) is set at 100% (membrane + cytosol) and the treatment value calculated in relation to the control. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

physiological conditions, so the biochemical studies must be carried out in whole muscle samples. Indeed, we show that M_2 signaling can affect post-synaptic proteins like PKCα. Thus, to better define the location of the presynaptic PKCβI and PKCε signaling in this study, we separated the synaptic-enriched region of the hemidiaphragms from the extrasynaptic regions. This can be done by adding α-bungarotoxin conjugated to TRITC to the medium at the last 10 minutes of treatment and then dissecting under the microscope the tissue region positive in AChRs (synaptic region) from the region without AChR-marking (extrasynaptic region) (Figure 8A). Although α-BTX-TRITC is added in a non-blocking low concentration, the results could be influenced by the presence of this nicotinic AChR blocker (see Discussion). As expected, PKCβI, PKCε, Munc18-1, SNAP-25, and their phosphorylated forms were enriched in the synaptic region and significantly lower in the extrasynaptic region (Figure 8B-F). The presence of these molecules in the extrasynaptic region could be due to their presence in axon branches, muscle spindles and, feasibly, distant or non-stained NMJs that cannot be excluded from the extrasynaptic region. M_2 blockade did not affect PKCβI phosphorylation or its total levels (Figure 8C). On the other hand, the effect of methoctramine over PKCε was restricted at the synaptic region, reducing PKCε phosphorylation and upregulating PKCε total protein levels (Figure 8D). The levels of PKCε at the extrasynaptic region were lower than at the synaptic region and methoctramine did not induce any effect. With regard to Munc18-1, methoctramine increased its protein levels and phosphorylation in the synaptic region (Figure 8E). Although Munc18-1 was enriched in the synaptic region of the diaphragm, this molecule was more abundant than the others in the extrasynaptic area (around 65% versus control values). In contrast, SNAP-25 was more specific for the synaptic region than Munc18-1 and, in concordance with the whole muscle samples, it was not affected

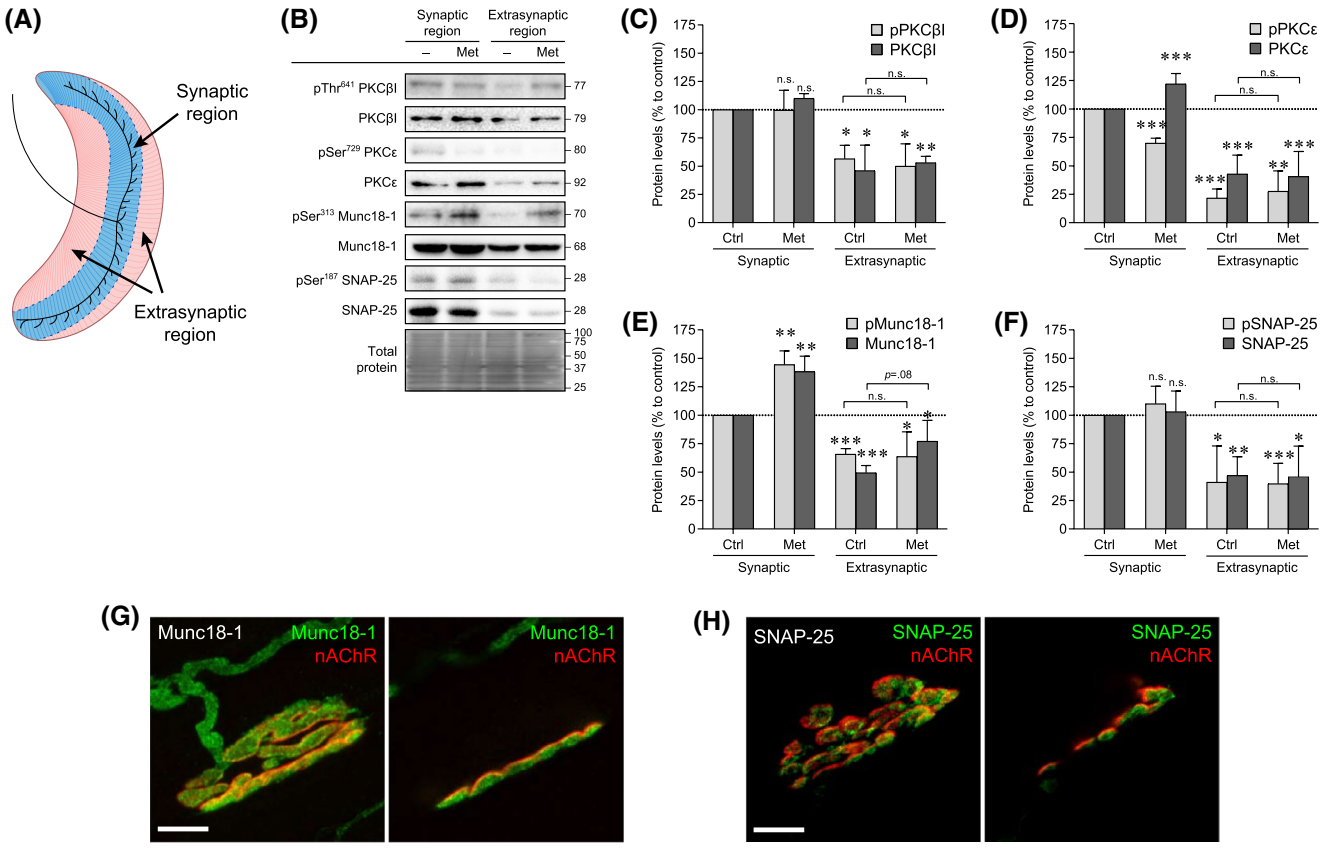

FIGURE 8 Synaptic localization of the effects of M_2 signaling. A, Representation of the synaptic and extrasynaptic regions of the rat diaphragm. B, Western blot bands of the phosphorylated and total levels of PKCβI, PKCε, Munc18-1, and SNAP-25 in the synaptic and extrasynaptic regions of the diaphragm before and after M_2 blockade (methoctramine, Met). C–F, Effect of M_2 blockade over the phosphorylation and total levels of (C) PKCβI, D, PKCε, (E) Munc18-1, and (F) SNAP-25. G–H, immunofluorescence-stained neuromuscular junctions of LAL muscle visualized at the confocal microscope. The images at the right are a confocal optical section of the left NMJ. NMJ with double labeling: α-bungarotoxin (α-BTX) conjugated with TRITC in red and Munc18-1 in green (G) and SNAP-25 in green (H). Scale bars = 10 μm. Data are expressed as percentage of protein levels before and after treatment (mean value ± SD). The control value (Ctrl) from the synaptic region is set at 100% and the rest are calculated in relation to the control. n.s. not significant, $*P < .05$, $**P < .01$, $***P < .001$

by methoctramine (Figure 8F). In concordance to the presence of Munc18-1 at the extrasynaptic region, Figure 8G shows that Munc18-1 is expressed at the presynaptic component of the NMJ and also in nerve terminals, which extend outside the synaptic region. The optical section shows that Munc18-1 immunostaining is concentrated at the presynaptic component, over the nAChR postsynaptic gutters (Figure 8G right). SNAP-25 was exclusively located in the presynaptic component of the NMJ (Figure 8H). The optical section shows that SNAP-25 green immunolabeling is concentrated over the nAChR postsynaptic gutters (Figure 8H right). Altogether, these results demonstrate the effect of M_2 blockade on PKCβI, PKCε, Munc18-1, and SNAP-25 is associated to the synaptic area of the diaphragm, reinforcing that this particular signaling occurs at the presynaptic terminal.

4 | DISCUSSION

The M_1 and M_2 muscarinic receptor subtypes induce opposed outcomes on ACh release at the NMJ. M_1 increases whereas M_2 decreases the end-plate potential.[1] Interestingly, both subtypes couple PKC to neurotransmission when inhibited with selective blockers such as pirenzepine or methoctramine. The M_1 and M_2 muscarinic receptors are specifically expressed at the nerve terminal and Schwann cells,[57] where they likely modify several PKC isoforms. Until now, it was unknown if muscarinic receptors had different preference for particular PKC isoforms. In this study, we selected the PKCβI and PKCε as representatives of classical and novel PKC families because they are exclusively expressed at the presynaptic terminal [21-23] and participate in synaptic transmission.[22,24,31,34,38] A summary of the main findings is represented in Figure 9.

4.1 | M_1 signaling on PKC

The M_1 muscarinic signaling potentiates ACh release at many cholinergic synapses, including the neuromuscular junction.[1,53,58] M_1 muscarinic receptors are linked to G_q protein signaling and PLCβ activation.[59] In turn, PLCβ activates PKC through the production of inositol 1,4,5-triphosphate (IP_3), which mobilizes Ca^{2+}, and the phorbol ester diacylglycerol.[60] Then, neurotransmitter release is enhanced through PKC, which phosphorylates numerous targets of the exocytotic machinery, but also by other phorbol ester-sensitive proteins like Munc13.[26,61,62]

Our results show that M_1 signaling promotes the maturation (priming) of both PKCβI and PKCε isoforms. This could

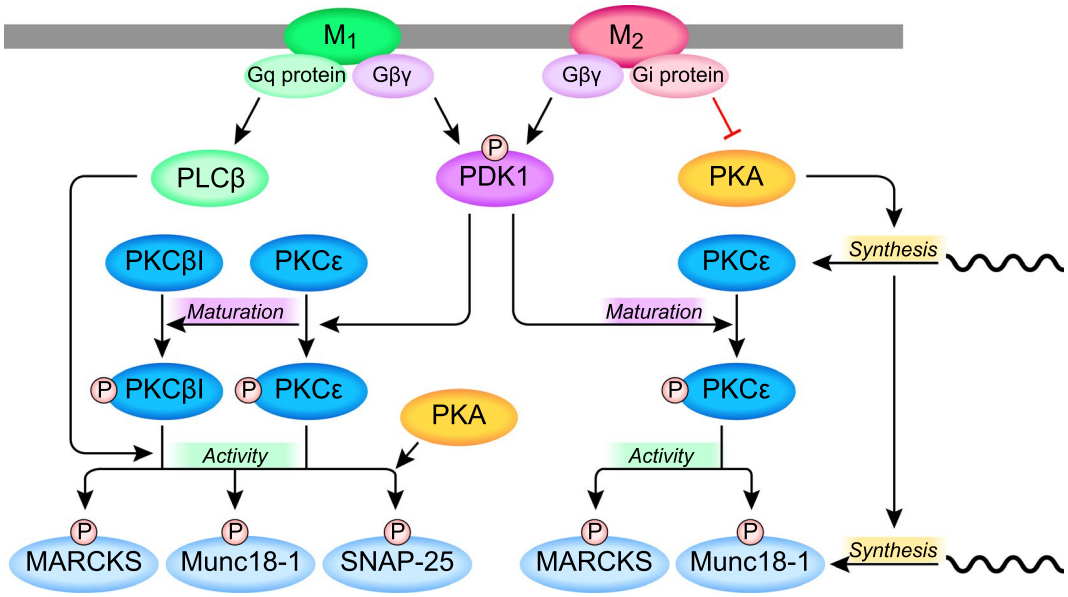

FIGURE 9 Summary of M_1 and M_2 muscarinic regulation of PKC signaling at the NMJ. The M_1 mAChR promotes the phosphorylation of PDK1 and the maturation of the presynaptic PKC isoforms PKCβI and PKCε at the membrane compartment of the NMJ. M_1 also activates PKC isoforms through PLCβ, causing PKCβI protein degradation at the membrane and PKCε release to the cytosol (see Discussion for the role of PDK1 on PKC translocation). This signaling pathway triggers the PKC phosphorylation of Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}), and MARCKS ($Ser^{152/156}$) and their recruitment to the membrane (SNAP-25 phosphorylation by M_1 requires PKA activity). In parallel to M_1, the M_2 mAChR signaling also promotes the phosphorylation of PDK1 and the priming of the PKC isoform PKCε at the membrane. However, M_2 downregulates PKCε protein levels at the membrane through a PKA-dependent pathway. This inhibition extends to the PKC substrates Munc18-1 and MARCKS. If the PKA-dependent pathway of M_2 is blocked, M_2 signaling is still able to promote PKCε maturation, which enhances Munc18-1 and MARCKS phosphorylation, similarly to M_1 signaling. Therefore, M_1 and M_2 muscarinic receptors balance PKCβI and PKCε priming, protein levels, and activity on the mediators of the synaptic vesicle release machinery

be mediated by PI3K/PDK1 because M_1 signaling activates PI3K in rat hippocampal neuron cultures[63] and we accordingly found that M_1 increases PDK1 Ser^{241} phosphorylation and PDK1 promotes both PKCβI and PKCε phosphorylations. On the other hand, M_1 mAChR regulates differently the levels of PKCβI and PKCε isoforms. It downregulates PKCβI in the total and in the membrane fraction but does not affect PKCε total protein levels and translocates it to the cytosol. Several reasons indicate that M_1 might be inducing PKCβI degradation after activation rather than inhibiting PKCβI synthesis. First, M_1 associates to the G_q protein and PKC activation, which leads to PKC activity-dependent degradation.[17-20] In concordance, we previously found at the NMJ that PKCβI degradation occurs after being activated by phrenic nerve stimulation to potentiate ACh release.[5,22] Another finding supporting that M_1 mAChR triggers PKCβI activity and its turnover is that the PKC downregulation occurs at the membrane fraction, the compartment where studies in cell cultures found activity-dependent PKCβI ubiquitination and degradation.[17] Additionally, we found that M_1 mAChR requires PKCβI activity to induce the phosphorylation of the SNARE regulator Munc18-1 (Ser^{313}), the SNARE core protein SNAP-25 (Ser^{187}) and the cytoskeleton-related MARCKS ($Ser^{152/156}$). In relation to Munc18-1 and PKCβI, experiments with the same βI inhibitor used here demonstrated that PKCβI is also necessary for the increase of Munc18-1 phosphorylation caused by nerve stimulation but not for the increase of Munc18-1 levels.[31] Also, the enhance of Munc18-1 PKC-phosphorylation is closely related to neurotransmission[26] and adds to our knowledge about how M_1 enhances acetylcholine release at the NMJ.[1] On the other hand, SNARE complex formation and neurotransmission is also regulated by SNAP-25 PKC-phosphorylation.[33,36,64] Our results show that M_1 mAChR uses PKCβI to phosphorylate SNAP-25, reinforcing the previously demonstrated role of the βI isoform on the neurotransmission at the NMJ as the inhibition of PKCβI decreases the size of end-plate potentials.[22] M_1 promotion of PKC phosphorylation over MARCKS has been determined in literature.[40] Multiple PKC isoforms can phosphorylate MARCKS in vitro and in vivo.[23,65] Here, we determine that, at the NMJ, M_1 requires the presynaptic PKCβI to phosphorylate MARCKS. Additionally, we found that the phosphorylation of Munc18-1, SNAP-25 and MARCKS by M_1 signaling occurs at the membrane and that M_1 also induces the translocation of Munc18-1 from the cytosol to the membrane. These changes in the membrane are concordant with an activation of all three substrates, which is closely related to their membrane association.[33,66,67]

Contrary to PKCβI, M_1 inhibition did not affect PKCε protein levels. This distinct behavior between PKCβI and PKCε could be because PKCε is less sensitive to activity-dependent degradation. Indeed, PKCε rate of downregulation is 3-fold slower than other PKC isotypes.[68] Alternatively, PKCε activity-dependent degradation can be modulated by other PKC isoforms.[69] In this regard, we know that the inhibition of PKCβI in basal conditions downregulates PKCε levels at the neuromuscular junction.[31] Thus, the blockade of M_1/PKCβI with pirenzepine could reduce PKCε and counter an accumulation of PKCε levels after M_1 inhibition. The PKCε isoform is crucial to facilitate and trigger multiple mechanisms involved in ACh release at the NMJ.[23,24,31,38] This isoform generally participates in the first signaling steps of multiple receptors, acting like an early kinase of various signaling cascades.[24] This is also the case for muscarinic signaling, where PKCε inhibition prevents mAChRs from modulating ACh release if it is performed before, but PKCε inhibition does not influence the NMJ release after mAChR blockade.[24] In concordance, here we inhibited PKCε before muscarinic blockade to demonstrate the dependency of M_1 on PKCε activity at the molecular level. Here we found that M_1 muscarinic signaling uses PKCε to phosphorylate the three substrates Munc18-1, SNAP-25, and MARCKS, demonstrating the facilitatory role of this kinase for M_1. In concordance, PKCε activity has been previously linked to the phosphorylation of Munc18-1,[31] SNAP-25,[38] and MARCKS.[23,65] The results also show that M_1 inhibition decreases the primed pPKCε form and enhances total PKCε in the membrane. We interpret this result as that, after M_1 inhibition, PKCε isoform is not allowed to perform its facilitating role and therefore it does not undergo activity-induced degradation at the membrane,[17-20,23,38] resulting in an accumulation of the inactive isoform in the membrane. Altogether, these molecular results support the previous electrophysiological findings showing that PKCε is necessary for M_1 to increase ACh release.[24] Because the membrane fraction that we studied contains the insoluble membrane compartments like the plasma membrane, synaptic vesicles, and mitochondria, it is possible that inhibited PKCε becomes more associated to the cytoskeleton (insoluble fraction)[70,71] or translocates between different cell compartments.[72] On the other hand, PKC priming by PDK1 acts on cytoskeleton-associated PKC enzymes (in the insoluble membrane fraction) and releases them to the cytosol.[70,71] Therefore, it is possible that the PDK1 inhibition by pirenzepine contributes to the association of total non-phosphorylated PKCε to the membrane fraction (in this case associated to the insoluble cytoskeleton). In addition, it should not be discarded that the increase of PKCε in the membrane after Pir could induce some activity on another substrate which we did not study here. Previous work at the NMJ suggested that the treatment with pirenzepine, although it downregulates IP_3/Ca^{2+} signaling and PKC activity, also couples at least some PKC isoform to participate in ACh release.[1] Our research drives us to think that this isoform could be PKCε acting on different p-substrates than Munc18-1, MARCKS, and

SNAP-25, as it seems to integrate different receptor signaling pathways at the NMJ[23,24,31,38] and here we find that it is recruited to the membrane. Together with our translocation data, the translocation of PKCε to the membrane after M_1 inhibition could be responsible for the coupling of PKC after M_1 inhibition. However, further research on the role of PKCε and its targets is needed to comprehend this complex mechanism. On the other hand, it is not surprising that both PKCβI and PKCε could phosphorylate these substrates, considering that all PKC isoforms show a high degree of sequence similarity in their kinase domain.[65] It is very interesting that both PKCβI and ε isoforms are necessary to phosphorylate these substrates, but they do not replace themselves (necessary but not sufficient). In coincidence, the activity of the novel PKCε isoform is a prerequisite necessary for classic PKC activity at the NMJ.[24] This remarks the importance of studying the differences and cooperation between PKC isoforms.

PKA activity is essential for maintaining neurotransmission at the NMJ and it has recently been linked with M_1 muscarinic signaling.[2,63] Here we show that M_1 signaling does not require PKA activity to promote the PKC phosphorylation of Munc18-1. However, PKA participates in Munc18-1 expression and we discuss it in the next section about M_2 signaling. In regard to MARCKS, PKA does not promote the M_1/PKC phosphorylation but it maintains its protein levels. This is because, without PKA activity, M_1 blockade caused a decrease in MARCKS levels. PKA is involved in protein translation and shutting down PKA from the system might be revealing a MARCKS-degrading pathway induced by M_1 mAChR inhibition. Interestingly, M_1/PKC phosphorylation of SNAP-25 Ser^{187} requires PKA activity. PKA is known to phosphorylate SNAP-25 on Thr^{138}.[37,73] We previously determined that blocking the neuromuscular PKA with H-89 decreases SNAP-25 Thr^{138} phosphorylation.[2] Together with the present results, this might indicate that PKA SNAP-25 Thr^{138} favors PKC phosphorylation on SNAP-25 Ser^{187}.

Therefore, at the neuromuscular junction, M_1 activates PDK1 and induces the maturation of the classical PKCβI and the novel PKCε. Besides, M_1 uses G_q/PLCβ and calcium mobilization to trigger PKCβI activity and its consequent degradation. M_1 needs PKCε activity to facilitate the M_1 downstream signaling. Both PKC isoforms regulate the phosphorylation of Munc18-1, MARCKS, and SNAP-25 substrates (the latter with the help of PKA), which associate to the membrane and participate in neurotransmission at the NMJ and other cellular processes.

4.2 | M_2 signaling on PKC

The M_2 muscarinic signaling reduces the neurotransmission from cholinergic synapses, including the neuromuscular junction.[1,53] M_2 receptors are generally linked to Gi proteins, which inhibit adenylate cyclase and block PKA activity by downregulating cAMP production.[58] One of the consequences we recently demonstrated is that M_2 signaling decreases the PKA phosphorylation of SNAP-25 Thr^{138} at the NMJ.[2] Besides the PKA pathway, further studies revealed that PKC activity is also necessary for the M_2 muscarinic signaling in various neuromuscular models.[1,74,75]

In the present work, we studied how M_2 influences the PKC isoforms that are exclusive of the presynaptic terminal.[21-23] In particular, we show that M_2 signaling does not affect the classical PKCβI isoform priming, levels or subcellular distribution and this PKC isoform is not required for any substrate phosphorylation affected by M_2 blockade that we studied. In concordance, a previous study in portal vein myocytes shows that M_2 recruits novel instead of classical PKC isoforms.[76] Our study concurs with this idea, because we found that the novel PKCε isoform participates in many M_2 downstream regulations at the NMJ.

M_2 signaling induces PKCε phosphorylation priming at the membrane fraction as well as the phosphorylation of the PKC master kinase PDK1, in the same way as M_1. This could be linked to M_2/PI3K activation through the Gβγ subunit[74,76] and here we demonstrate that PDK1 promotes PKCε phosphorylation. On the other hand, M_2 signaling downregulates PKCε protein levels in the membrane fraction. These modulations of PKCε and pPKCε by M_2 occur at the synaptic region of the hemidiafragm, in consistency with the presynaptic location of the isoform.[21,23] As mentioned before, the increase of PKC levels can be interpreted as an accumulation of the inactive kinase or, on the contrary, as more synthesis. M_2 activity is unlikely to induce PKCε activity and degradation, as the PKCε isoform is not very sensitive to activity-dependent degradation[68] and M_2 inhibition activates and couples PKC to participate in acetylcholine release at the NMJ.[1] Besides, evidence demonstrate that PKCε is active. We observed that M_2 blockade induces PKC activity, upregulating Munc18-1 PKC-phosphorylation, Munc18-1 protein levels and MARCKS PKC-phosphorylation. In concordance, PKCε activity at the NMJ upregulates both Munc18-1 phosphorylation and protein levels[31] and phosphorylates MARCKS.[23] Also, here we verified that PKCε activity is required for M_2 blockade to upregulate Munc18-1 and MARCKS. Thus, the increase in PKCε by M_2 inhibition is consistent with enhanced PKC activity and it is possibly caused through protein synthesis (see below for the implication of PKA).

To identify the components of M_2/PKCε pathway, we investigated the effect of PKA inhibition. Our results show that M_2 blockade uses PKA activity to increase PKCε levels but does not need PKA to decrease PKCε phosphorylation. This reveals that M_2 signaling affects PKCε through two different pathways: a PKA-dependent pathway where M_2 downregulates PKCε, and a PKA-independent pathway where

M_2 induces PKCε maturation through phospho-PDK1. The mentioned PKA-independent pathway is concordant with the previous studies linking M_2 and novel PKC through the Gβγ subunit and PI3K activation,[74,76] hence does not need G_i protein and PKA activity. On the other hand, M_2 reduces PKCε levels via G_i protein and PKA inhibition and, when M_2 is blocked, PKA activity increases and upregulates PKCε. The phosphorylation of PKCε does not increase along with the total level because the PDK1-induced phosphorylation remains downregulated by Met. Because the PKCε isoform is exclusively expressed at the synaptic terminal of NMJs,[24] where gene expression does not take place, the upregulation of its protein levels might be caused by a posttranscriptional mechanism. Concordantly, PKA activity has been associated to the modulation of mRNA translation through the phosphorylation of the eukaryotic elongation factor 2 kinase, inducing a general reduction of mRNA translation but increasing the translation rate of a small subset of synaptic proteins.[77,78] Also, PKA promotes the translation of various proteins via phosphorylation of polypyrimidine tract-binding protein 1.[79]

Regarding the span of M_2 signaling, this mAChR subtype is specifically expressed at the nerve terminal and Schwann cells.[57] Here we describe a molecular pathway confined in the nerve endings, because the participating PKCβI and PKCε are exclusively presynaptic[5,22,23] and the end-targets are Munc18-1 and SNAP-25, neurotransmision modulators unique to the nerve endings.[31,38] We observed that M_2 signaling only affects the synaptic region of the hemidiaphragm, where both PKC isoforms, Munc18-1 and SNAP-25 were enriched. Munc18-1 was quite detectable in the extrasynaptic area (around 65% versus control value). This is in line with our previous report that Munc18-1 is also present in the nerve axons,[31] which stretch outside the synaptic region. In fact, when studying the effect of methoctramine in the extrasynaptic region, we observed a tendency to increase Munc18-1 protein levels ($P = .8$) that was not significant. However, muscarinic signaling may extend further than the presynaptic terminal and affect postsynaptic proteins. Here we show that M_2 mAChR downregulates the levels and priming of PKCα, an isoform which is mainly expressed at the postsynaptic site. M_2 mAChR at the NMJ induces an auto-inhibitory feedback over ACh release and our data suggest that M_2 signaling on PKCα could be linked to a reduction of the postsynaptic responsiveness to ACh. Other findings showing that synaptic events influence pre- and post-synaptic kinases are that mAChR signaling controls the widely expressed PKA[2] and that phrenic-induced activity under blocked postsynaptic contraction induces the degradation of the presynaptic PKCβI and the post-synaptic PKCβII.[5]

In this study we found that M_2 does not affect SNAP-25 Ser^{187} phosphorylation by PKC at the NMJ. Indeed, M_2 signaling rather regulates SNAP-25 through the PKA-phosphorylation at Thr^{138}.[2] Here, we also demonstrate that PKA activity is required for M_2 to modulate Munc18-1 and MARCKS. Interestingly, we found that all PKC phosphorylations inhibited by M_2, and hence attributed to PKCε, require PKA activity. This further supports the idea that M_2/PKA pathway modulates upstream the activity of PKCε over those substrates,[23,31] which provide a molecular clue to the functional results about the PKA-dependent pathway on M_2 outcomes.[80] Interestingly, we also found that a previous inhibition of PKA shifts M_2 to perform an M_1-like signaling in relation to Munc18-1 and MARCKS phosphorylation. This is because after H89 preincubation, M_2 blockade decreases both Munc18-1 and MARCKS phosphorylation which is the result of M_1 inhibition. This is concordant with previous functional studies that showed when PKA is previously inactivated, blocking either M_1 or M_2 leads to a similar reduction in transmitter release.[1] Indeed, this result unmasks the PKA-independent pathway described before where M_2 induces PKCε phosphorylation. In this, M_2 blockade would downregulate PKCε phosphorylation through PDK1 (our results) and Gβγ/PI3K[74,76] and, without PKA activity in the system, this PKC inhibition would decrease Munc18-1 and MARCKS phosphorylation.

4.3 | Muscarinic balance on PKC at the NMJ

We examined the joint action of M_1 and M_2 receptors with the pan-muscarinic inhibitor atropine. We found that atropine affects PKCε but not PKCβI, a similar effect to inhibit M_2 with the selective inhibitor methoctramine. Atropine's affinity is practically the same for both M_1 and M_2 mammalian subtypes,[58] which could indicate that M_2 inhibition has a greater overall effect on the protein levels of PKC than M_1. Concordantly, previous studies showed that atropine increases ACh release at the NMJ in the same way as methoctramine.[1,53] Alternatively, the fact that atropine does not replicate the effect of M_1 inhibition on PKCβI protein levels might indicate that M_1 needs an active M_2 to modulate PKCβI turnover.

The upregulation of PKCε by atropine can be explained by the opposite action of M_1 and M_2. On the one hand, atropine blocks M_1 thus decreasing PKCε activity and the phosphorylation of its substrate Munc18-1 (discussed in more detail below). However, the accompanying M_2 blockade by atropine also liberates PKA activity, which upregulates PKCε levels and buffers the action of M_1. This M_1/M_2 counter regulation probably balances PKCε activity and protects the synapse from an excessive or insufficient PKC activity.

Regarding PKC priming, atropine decreases the phosphorylation of the upstream kinase PDK1, indicating that the overall action of M_1 and M_2 muscarinic receptors activates PDK1 at the NMJ. Interestingly, atropine causes the

same decrease in PDK1 levels as the decrease observed when M_1 or M_2 are inhibited separately. The lack of additive effect suggests that both mAChR subtypes could be using the same pathway. As mentioned before, both M_1 and M_2 muscarinic receptors have been shown to activate PI3K,[63,74,76] which could be a common mechanism to activate PDK1. We previously demonstrated that PKC priming phosphorylation at the NMJ is enhanced by presynaptic stimulation and that the resulting muscle contraction increases it further,[22] suggesting a postsynaptic retrograde regulation. However, tropomyosin-related kinase B (TrkB) receptor signaling was not responsible for this priming and, contrarily, it rather acted downregulating PKCα and PKCβI phosphorylation levels.[22] That suggested the existence of a different pathway, activated by neuromuscular activity, which was promoting PKC phosphorylation and compensating TrkB downregulation. In the current study, we found that M_1 and M_2 muscarinic receptors promote PDK1 activity as well as PKC maturation at the NMJ. These receptors, whose action is triggered by the ACh released at the NMJ, could explain how neuromuscular activity enhances PKC maturation in an activity-dependent manner.

Atropine action over the PKC activity can be observed by studying PKC substrates. In this study we observed that atropine downregulates Munc18-1 phosphorylation, increases Munc18-1 protein levels, and it does not affect the levels or phosphorylation of SNAP-25 and MARCKS. Comparing these results with the selective inhibitions suggests that M_1 blockade is responsible for the decrease in phospho Munc18-1, via PKCβI and PKCε, whereas M_2 blockade is responsible for the increase in Munc18-1 protein levels, via PKCε and PKA. Therefore, the activities of both mAChRs balance each other: M_1 promotes Munc18-1 phosphorylation whereas M_2 signaling controls the levels of this regulatory synaptic protein. Regarding SNAP-25, it is a key synaptic molecule which is difficult to modulate with treatments due to the multiple mechanisms finely regulating it.[2,37,38,81] Interestingly, here we show that M_1 blockade decreases SNAP-25 Ser^{187} phosphorylation, although we could not observe this effect with the general muscarinic inhibitor atropine. This suggests that M_1 signaling needs M_2 active to promote SNAP-25 PKC-phosphorylation. Interestingly, the PKA phosphorylation of SNAP-25 (Thr^{138}) follows a similar regulation, where M_2 downregulates it and needs the activity of M_1.[2] If the inhibitory M_2 signaling on PKA/SNAP-25 is necessary for the M_1/PKC phosphorylation of SNAP-25, the current results might indicate that PKA hinders the action of PKC over SNAP-25. However, further research is needed to shed light on this complex mechanism and clarify the complementary role of PKC and PKA on SNAP-25. Regarding the third substrate examined, the absence of effect over MARCKS phosphorylation is probably the result of the counter regulation between M_1 and M_2: the decreasing effect of M_1 blockade and the increasing effect of M_2 blockade probably cancel each other out. MARCKS phosphorylation is related to PKCε[23] and, concordantly, here we demonstrated that both mAChR subtypes modulate PKCε and require its activity to modulate MARCKS.

As a pan-muscarinic inhibitor, atropine also inhibits other mAChR subtypes like M_3, M_4 and M_5. However, studies at the adult NMJ show that mainly M_1 and M_2 orchestrate the NMJ neurotransmitter release,[53,82-84] whereas other mAChR subtypes like M_3 and M_4 only participate during the development of the newborn NMJ.[83,85,86] M_1 and M_2 implication can be determinated with a mixture of Pir+Met. In the frog NMJ, this mixture fully mimmicks the effects of atropine on neurotransmitter release[53] and in rat hippocampal place cells, where the mixture replicated the effects of scopolamine, another known pan-muscarinic inhibitor.[87] As expected, we observed that Pir + Met incubation causes the same effects as atropine over PKCβI, Munc18-1, SNAP-25, and MARCKS protein levels and phosphorylation, reinforcing the major participation of M_1 and M_2 in the muscarinic signaling.

Finally, the role of PDK1 activity at the NMJ also demonstrates the balance between M_1 and M_2 receptors. Overall, PDK1 blockade per se has effects similar to M_1 inhibition, regulating in an equal manner PKCβI, PKCε, and the substrates SNAP-25 and MARCKS. This supports that M_1 signaling relies mainly on the PKC pathway, where PDK1 activity plays an important role. On the other hand, M_2 blockade also inhibits PDK1 activity and we observed that PDK1 blockade per se induces effects similar to M_2 inhibition over Munc18-1 phosphorylation. Interestingly, atropine does not induce the same modulations as PDK1 inhibition. This is probably because PDK1 inhibition only disrupts PKC priming, one step of PKC activation, whereas atropine induces a pan-muscarinic inhibition, involving both the PKC pathway—including PDK1—and the PKA pathway.

4.4 | Conclusion and future prospects

The present results demonstrate a signaling pathway that M_1 and M_2 mAChRs use to regulate neurotransmission. The M_1 mAChR signaling promotes the phosphorylation of PDK1 and the priming of the presynaptic PKC isoforms PKCβI and PKCε at the NMJ, which occurs at the membrane compartment. On the same subcellular compartment, M_1 activation of PKC induces PKCβI protein degradation and displaces PKCε from the membrane, without changing PKCε total protein levels. This signaling pathway triggers the PKC phosphorylation of Munc18-1 (Ser^{313}), SNAP-25 (Ser^{187}), and MARCKS ($Ser^{152/156}$) and their recruitment to the membrane. On the other hand, the M_2 mAChR signaling also promotes the phosphorylation of PDK1 and the priming of PKCε at the membrane compartment. However, M_2

signaling downregulates PKCε protein levels at the membrane compartment through a PKA-dependent pathway. This inhibition extends to the PKC substrates Munc18-1 and MARCKS. Interestingly, when PKA is blocked, M_2 signaling is able to promote Munc18-1 and MARCKS phosphorylation like M_1 signaling. The complementary activities of M_1 and M_2 muscarinic receptors balance PKCβI and PKCε priming, protein levels, and activity on mediators of the synaptic vesicle release machinery. Altogether this provides for the first time a molecular clue of M_1 and M_2 muscarinic and PDK1/PKC regulation of neurotransmitter release at the NMJ.

In this work we have identified a signaling pathway specific of the presynaptic motoneuron. This has been possible because we selected PKC isoforms and targets that participate in neurotransmitter release and are exclusively expressed at the presynaptic terminal of the NMJ.[21-23] We performed several tests to verify the location of the signaling in this study: (i) we confirmed by immunohistochemistry the presynaptic location of these molecules at the NMJ, (ii) we demonstrated that the effect of M_2 blockade on PKCβI, PKCε, Munc18-1, and SNAP-25 is associated to the synaptic area of the diaphragm, reinforcing that this particular signaling occurs at the presynaptic terminal, and (iii) we checked the effect of mAChR antagonists over the PKCα isoform, which is preferentially expressed in the postsynaptic muscles, showing the communication between cells through the mAChR signaling and the complexity of the mAChR regulation in the tripartite cellular NMJ.

Altogether, our observations provide in vivo examples of muscarinic modulation in a physiological model. Identifiying how muscarinic inhibitors and PKC isoforms participate in neurotransmission is important for prospective therapies. For instance, drugs in development depend upon the balance between the various isoenzymes present.[88] PKC isoforms are key for neurotransmitter release at the NMJ and are affected in symptomatic and presymptomatic stages of amyotrophic lateral sclerosis.[89,90] The dual modulation of M_1 and M_2 mAChRs could be used to readjust neuromuscular function and preserve neuromuscular function and muscle strength being useful for muscular paralysis, fall prevention, aging, and neuromuscular disorders such as amyotrophic lateral sclerosis and Duchenne muscular dystrophy.

ACKNOWLEDGMENTS

This work was funded by two grants from the Ministerio de Economía y Competitividad (MINECO), the Agencia Estatal de Investigación (AEI), and the European Regional Development Fund (ERDF): (SAF2015-67143-P and PID2019-106332GB-I00); a grant from the Universitat Rovira i Virgili (URV) (2017PFR-URV-B2-85); and a grant from the Catalan Government (2017SGR704). VC and MD were supported by a grant from MINECO under the framework of the Sistema Nacional de Garantía Juvenil, the European Social Fund (ESF) and the Iniciativa de Empleo Juvenil (IEJ) reference codes: LE1511314-2014PEJ-04 and LE1911587-2019PEJ-04. AP was supported by the Spanish Ministerio de Ciencia Innovación y Universidades (Grant no. PRE2020-092084, project no. PID2019-106332GB-I00). LJ was supported by the Universitat Rovira i Virgili (URV) under the framework of the "Programa Martí i Franquès d'ajuts a la investigació. Contractes de personal investigador predoctoral en formació (2018PMF-PIPF-18)".

CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICS STATEMENT

The animals were cared for in accordance with the guidelines of the European Community Council Directive of 24 November 1986 (86/609/EEC) for the humane treatment of laboratory animals. All the procedures realized were revised and authorized by the Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 0289).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MAL, JT, NG. Performed the experiments: VC, LJ, AP, MD, MT. Analyzed the data: VC, MAL, JT. Wrote the paper: VC, NG, MAL, JT.

ORCID

V. Cilleros-Mañé https://orcid.org/0000-0001-5690-9932
L. Just-Borràs https://orcid.org/0000-0003-0473-3730
A. Polishchuk https://orcid.org/0000-0001-6445-1538
M. Durán https://orcid.org/0000-0003-1321-6305
M. Tomàs https://orcid.org/0000-0002-4151-1697
N. Garcia https://orcid.org/0000-0002-3401-8335
J. M. Tomàs https://orcid.org/0000-0002-0406-0006
M. A. Lanuza https://orcid.org/0000-0003-4795-4103

REFERENCES

1. Santafé MM, Lanuza MA, Garcia N, Tomàs M, Tomàs JM. Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal. *Neuroscience*. 2007;148:432-440.
2. Cilleros-Mañé V, Just-Borràs L, Tomàs M, Garcia N, Tomàs JM, Lanuza MA. The M2 muscarinic receptor, in association to M1, regulates the neuromuscular PKA molecular dynamics. *FASEB J*. 2020;34:4934-4955.
3. Lanuza MA, Santafé MM, Garcia N, et al. Protein kinase C isoforms at the neuromuscular junction: Localization and specific roles in neurotransmission and development. *J Anat*. 2014;224:61-73.
4. Nagy G, Matti U, Nehring RB, et al. Protein kinase C-dependent phosphorylation of synaptosome-associated protein of 25 kDa at Ser187 potentiates vesicle recruitment. *J Neurosci*. 2002;22:9278-9286.

5. Besalduch N, Tomàs M, Santafé MM, Garcia N, Tomàs J, Lanuza MA. Synaptic activity-related classical protein kinase C isoform localization in the adult rat neuromuscular synapse. *J Comp Neurol*. 2010;518:211-228.
6. Newton AC. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem J*. 2003;370:361-371.
7. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. *EMBO J*. 2000;19:496-503.
8. Dutil EM, Toker A, Newton AC. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr Biol*. 1998;8:1366-1375.
9. Cazaubon SM, Parker PJ. Identification of the phosphorylated region responsible for the permissive activation of protein kinase C. *J Biol Chem*. 1993;268:17559-17563.
10. Oancea E, Meyer T. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell*. 1998;95:307-318.
11. Violin JD, Zhang J, Tsien RY, Newton AC. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol*. 2003;161:899-909.
12. Osto E, Kouroedov A, Mocharla P, et al. Inhibition of protein kinase Cβ prevents foam cell formation by reducing scavenger receptor A expression in human macrophages. *Circulation*. 2008;118:2174-2182.
13. Wang X.Q., Yan Q, Sun P, et al. Suppression of epidermal growth factor receptor signaling by protein kinase C-activation requires CD82, caveolin-1, and ganglioside. *Cancer Res*. 2007;67:9986-9995.
14. Zhou J, Fariss RN, Zelenka PS. Synergy of epidermal growth factor and 12(S)-hydroxyeicosatetraenoate on protein kinase C activation in lens epithelial cells. *J Biol Chem*. 2003;278:5388-5398.
15. Hurtado E, Cilleros V, Just L, et al. Synaptic activity and muscle contraction increases PDK1 and PKCβI phosphorylation in the presynaptic membrane of the neuromuscular junction. *Front Mol Neurosci*. 2017;10. https://doi.org/10.3389/fnmol.2017.00270
16. Kraft AS, Anderson WB, Cooper HL, Sando JJ. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J Biol Chem*. 1982;257:13193-13196.
17. Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA. Activation of protein kinase C triggers its ubiquitination and degradation. *Mol Cell Biol*. 1998;18:839-845.
18. Lee H-W, Smith L, Pettit GR, Vinitsky A, Smith JB. Ubiquitination of protein kinase C-alpha and degradation by the proteasome. *J Biol Chem*. 1996;271:20973-20976.
19. Kang BS, French OG, Sando JJ, Hahn CS. Activation-dependent degradation of protein kinase C eta. *Oncogene*. 2000;19:4263-4272.
20. Parker PJ, Bosca L, Dekker L, Goode NT, Hajibagheri N, Hansra G. Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation. *Biochem Soc Trans*. 1995;23:153-155.
21. Perkins GA, Wang L, Huang LJ, et al. PKA, PKC, and AKAP localization in and around the neuromuscular junction. *BMC Neurosci*. 2001;2:17.
22. Hurtado E, Cilleros V, Nadal L, et al. Muscle contraction regulates BDNF/TrkB signaling to modulate synaptic function through presynaptic cPKCα and cPKCβI. *Front Mol Neurosci*. 2017;10:1-22.
23. Obis T, Besalduch N, Hurtado E, et al. The novel protein kinase C epsilon isoform at the adult neuromuscular synapse: location, regulation by synaptic activity-dependent muscle contraction through TrkB signaling and coupling to ACh release. *Mol Brain*. 2015;8:8.
24. Obis T, Hurtado E, Nadal L, et al. The novel protein kinase C epsilon isoform modulates acetylcholine release in the rat neuromuscular junction. *Mol Brain*. 2015;8:80.
25. Fujita Y, Sasaki T, Fukui K, et al. Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J Biol Chem*. 1996;271:7265-7268.
26. Barclay JW, Craig TJ, Fisher RJ, et al. Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *J Biol Chem*. 2003;278:10538-10545.
27. Morgan A, Craig TJ, Evans GJO. Use of phospho-specific antibodies to demonstrate phosphorylation of Munc18 / nSec1 in chromaffin cells. *Cell Biol Chromaffin Cell*. 2004;1:65-70.
28. Snyder DA, Kelly ML, Woodbury DJ. SNARE complex regulation by phosphorylation. *Cell Biochem Biophys*. 2006;45:111-123.
29. Sons MS, Verhage M, Plomp JJ. Role of Munc18-1 in synaptic plasticity at the myasthenic neuromuscular junction. *Ann N Y Acad Sci*. 2003;998:404-406.
30. Südhof TC. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron*. 2013;80:675-690.
31. Simó A, Just-Borràs L, Cilleros-Mañé V, et al. BDNF-TrkB signaling coupled to nPKCε and cPKCβI modulate the phosphorylation of the exocytotic protein Munc18-1 during synaptic activity at the neuromuscular junction. *Front Mol Neurosci*. 2018;11:207.
32. Shimazaki Y, Nishiki T, Omori A, et al. Phosphorylation of 25-kDa synaptosome-associated protein. Possible involvement in protein kinase C-mediated regulation of neurotransmitter release. *J Biol Chem*. 1996;271:14548-14553.
33. Kataoka M, Kuwahara R, Iwasaki S, Shoji-Kasai Y, Takahashi M. Nerve growth factor-induced phosphorylation of SNAP-25 in PC12 cells. *J Neurochem*. 2000;74:2058-2066.
34. Morgan A, Burgoyne RD, Barclay JW, et al. Regulation of exocytosis by protein kinase C. *Biochem Soc Trans*. 2005;33:1341-1344.
35. Yang Y, Craig TJ, Chen X, et al. Phosphomimetic mutation of Ser-187 of SNAP-25 increases both syntaxin binding and highly Ca^{2+}-sensitive exocytosis. *J Gen Physiol*. 2007;129:233-244.
36. Katayama N, Yamamori S, Fukaya M, et al. SNAP-25 phosphorylation at Ser187 regulates synaptic facilitation and short-term plasticity in an age-dependent manner. *Sci Rep*. 2017;7:7996.
37. Leenders AGM, Sheng Z-H. Modulation of neurotransmitter release by the second messenger-activated protein kinases: implications for presynaptic plasticity. *Pharmacol Ther*. 2005;105:69-84.
38. Simó A, Cilleros-Mañé V, Just-Borràs L, et al. nPKCε mediates SNAP-25 phosphorylation of Ser-187 in basal conditions and after synaptic activity at the neuromuscular junction. *Mol Neurobiol*. 2019;56:5346-5364.
39. Aderem A. The Marcks brothers: A family of protein kinase C substrates. *Cell*. 1992;71:713-716.
40. Willets JM, Nelson CP, Nahorski SR, Challiss RAJ. The regulation of M1 muscarinic acetylcholine receptor desensitization by synaptic activity in cultured hippocampal neurons. *J Neurochem*. 2007;103:2268-2280.
41. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM. Protein kinase C- ξ is responsible for the protection of preconditioning in rabbit cardiomyocytes. *J Mol Cell Cardiol*. 1999;31:1937-1948.
42. Zhang Y, Ying J, Jiang D, et al. Urotensin-II receptor stimulation of cardiac L-type Ca^{2+} channels requires the βγ subunits of Gi/o-protein and phosphatidylinositol 3-kinase-dependent protein kinase C β1 isoform. *J Biol Chem*. 2015;290:8644-8655.

43. Johnson JA, Gray MO, Chen CH, Mochly-Rosen D. A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J Biol Chem*. 1996;271:24962-24966.
44. Stebbins EG, Mochly-Rosen D. Binding specificity for RACK1 resides in the V5 region of βII protein kinase C. *J Biol Chem*. 2001;276:29644-29650.
45. Mochly-Rosen D, Gordon AS. Anchoring proteins for protein kinase C: a means for isozyme selectivity. *FASEB J*. 1998;12:35-42.
46. Way KJ, Chou E, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci*. 2000;21:181-187.
47. Di-Capua N, Sperling O, Zoref-Shani E. Protein kinase C-ε is involved in the adenosine-activated signal transduction pathway conferring protection against ischemia-reperfusion injury in primary rat neuronal cultures. *J Neurochem*. 2003;84:409-412.
48. Khasar SG, Lin YH, Martin A, et al. A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron*. 1999;24:253-260.
49. Najafov A, Sommer EM, Axten JM, DeYoung MP, Alessi DR. Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. *Biochem J*. 2011;433:357-369.
50. Ravichandran V, Chawla A, Roche PA. Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J Biol Chem*. 1996;271(23):13300-13303.
51. Rapaport D, Lugassy Y, Sprecher E, Horowitz M. Loss of SNAP29 impairs endocytic recycling and cell motility. *PLoS One*. 2010;5:e9759.
52. Iwasaki S, Kataoka M, Sekiguchi M, Shimazaki Y, Sato K, Takahashi M. Two distinct mechanisms underlie the stimulation of neurotransmitter release by phorbol esters in clonal rat pheochromocytoma PC12 cells. *J Biochem*. 2000;128:407-414.
53. Slutsky I, Parnas H, Parnas I. Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction. *J Physiol*. 1999;514:769-782.
54. Nakano S, Shimohama S, Saitoh T, Akiguchi I, Kimura J. Localization of protein kinase C in human skeletal muscle. *Muscle Nerve*. 1992;15:496-499.
55. Martinez-Pena y Valenzuela I, Pires-Oliveira M, Akaaboune M. PKC and PKA regulate AChR dynamics at the neuromuscular junction of living mice. *PLoS One*. 2013;8:e81311.
56. Steinberg SF. Structural basis of protein kinase C isoform function. *Physiol Rev*. 2008;88:1341-1378.
57. Garcia N, Santafé MM, Salon I, Lanuza MA, Tomàs J. Expression of muscarinic acetylcholine receptors (M1-, M2-, M3- and M4-type) in the neuromuscular junction of the newborn and adult rat. *Histol Histopathol*. 2005;20:733-743.
58. Caulfield MP, Birdsall NJ. International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev*. 1998;50:279-290.
59. Ross EM, Berstein G. Regulation of the M1 muscarinic receptor-Gq-phospholipase C-beta pathway by nucleotide exchange and GTP hydrolysis. *Life Sci*. 1993;52:413-419.
60. Newton AC. Regulation of protein kinase C. *Curr Opin Cell Biol*. 1997;9:161-167.
61. Lou X, Korogod N, Brose N, Schneggenburger R. Phorbol esters modulate spontaneous and Ca^{2+}-evoked transmitter release via acting on both Munc13 and protein kinase C. *J Neurosci*. 2008;28:8257-8267.
62. Wierda KDB, Toonen RFG, de Wit H, Brussaard AB, Verhage M. Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. *Neuron*. 2007;54:275-290.
63. Zhao L-X, Ge Y-H, Li J-B, et al. M1 muscarinic receptors regulate the phosphorylation of AMPA receptor subunit GluA1 via a signaling pathway linking cAMP-PKA and PI3K-Akt. *FASEB J*. 2019. https://doi.org/10.1096/fj.201802351R
64. Shu Y, Liu X, Yang Y, Takahashi M, Gillis KD. Phosphorylation of SNAP-25 at Ser187 mediates enhancement of exocytosis by a phorbol ester in INS-1 cells. *J Neurosci*. 2008;28:21-30.
65. Herget T, Oehrlein SA, Pappin DJC, Rozengurt E, Parker PJ. The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. *Eur J Biochem*. 1995;233:448-457.
66. de Vries KJ, Geijtenbeek A, Brian EC, de Graan PN, Ghijsen WE, Verhage M. Dynamics of munc18-1 phosphorylation/dephosphorylation in rat brain nerve terminals. *Eur J Neurosci*. 2000;12:385-390.
67. Disatnik MH, Boutet SC, Pacio W, et al. The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. *J Cell Sci*. 2004;117:4469-4479.
68. Olivier AR, Parker PJ. Identification of multiple PKC isoforms in Swiss 3T3 cells: Differential down-regulation by phorbol ester. *J Cell Physiol*. 1992;152:240-244.
69. Goode NT, Hajibagheri MAN, Parker PJ. Protein kinase C (PKC)-induced PKC down-regulation. *J Biol Chem*. 1995;270:2669-2673.
70. Newton AC. Protein kinase C: poised to signal. *Am J Physiol Endocrinol Metab*. 2010;298:E395-402.
71. Denning MF. Specifying protein kinase C functions in melanoma. *Pigment Cell Melanoma Res*. 2012;25:466-476.
72. Shirai Y, Sakai N, Saito N. Subspecies-specific targeting mechanism of protein kinase C. *Jpn J Pharmacol*. 1998;78:411-417.
73. Hepp R, Cabaniols J-P, Roche PA. Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A. *FEBS Lett*. 2002;532:52-56.
74. Wang Y-X, Dhulipala PDK, Li L, Benovic JL, Kotlikoff MI. Coupling of M 2 muscarinic receptors to membrane ion channels via phosphoinositide 3-Kinase γ and atypical protein kinase C. *J Biol Chem*. 1999;274:13859-13864.
75. Shen A, Mitchelson F. Muscarinic M2 receptor-mediated contraction in the guinea pig taenia caeci. *Biochem Pharmacol*. 1998;56:1529-1537.
76. Callaghan B, Koh SD, Keef KD. Muscarinic M2 receptor stimulation of Cav1.2b requires phosphatidylinositol 3-kinase, protein kinase C, and c-Src. *Circ Res*. 2004;94:626-633.
77. Heise C, Gardoni F, Culotta L, di Luca M, Verpelli C, Sala C. Elongation factor-2 phosphorylation in dendrites and the regulation of dendritic mRNA translation in neurons. *Front Cell Neurosci*. 2014;8. https://doi.org/10.3389/fncel.2014.00035
78. Chotiner J, Khorasani H, Nairn A, O'Dell T, Watson J. Adenylyl cyclase-dependent form of chemical long-term potentiation triggers translational regulation at the elongation step. *Neuroscience*. 2003;116:743-752.
79. Knoch K-P, Meisterfeld R, Kersting S, et al. cAMP-dependent phosphorylation of PTB1 promotes the expression of insulin secretory granule proteins in β cells. *Cell Metab*. 2006;3:123-134.
80. Santafé MM, Garcia N, Lanuza MA, Tomàs M, Tomàs J. Interaction between protein kinase C and protein kinase A can

modulate transmitter release at the rat neuromuscular synapse. *J Neurosci Res*. 2009;87:683-690.
81. Sørensen JB, Nagy G, Varoqueaux F, et al. Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell*. 2003;114:75-86.
82. Minic J, Molgó J, Karlsson E, Krejci E. Regulation of acetylcholine release by muscarinic receptors at the mouse neuromuscular junction depends on the activity of acetylcholinesterase. *Eur J Neurosci*. 2002;15:439-448.
83. Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomàs J. Muscarinic autoreceptors related with calcium channels in the strong and weak inputs at polyinnervated developing rat neuromuscular junctions. *Neuroscience*. 2004;123:61-73.
84. Wright MC, Potluri S, Wang X, et al. Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. *J Neurosci*. 2009;29:14942-14955.
85. Nadal L, Garcia N, Hurtado E, et al. Presynaptic muscarinic acetylcholine autoreceptors (M1, M2 and M4 subtypes), adenosine receptors (A1 and A2A) and tropomyosin-related kinase B receptor (TrkB) modulate the developmental synapse elimination process at the neuromuscular junction. *Mol Brain*. 2016;9:67.
86. Nadal L, Garcia N, Hurtado E, et al. Presynaptic muscarinic acetylcholine receptors and TrkB receptor cooperate in the elimination of redundant motor nerve terminals during development. *Front Aging Neurosci*. 2017;9. https://doi.org/10.3389/fnagi.2017.00024
87. Brazhnik E. The effects on place cells of local scopolamine dialysis are mimicked by a mixture of two specific muscarinic antagonists. *J Neurosci*. 2004;24:9313-9323.
88. Ersvaer E, Kittang AO, Hampson P, et al. The protein kinase C agonist PEP005 (ingenol 3-angelate) in the treatment of human cancer: a balance between efficacy and toxicity. *Toxins (Basel)*. 2010;2:174-194.
89. Just-Borràs L, Hurtado E, Cilleros-Mañé V, et al. Overview of impaired BDNF signaling, their coupled downstream serine-threonine kinases and SNARE/SM complex in the neuromuscular junction of the amyotrophic lateral sclerosis model SOD1-G93A mice. *Mol Neurobiol*. 2019;56:6856-6872.
90. Lanuza MA, Just-Borràs L, Hurtado E, et al. The impact of kinases in amyotrophic lateral sclerosis at the neuromuscular synapse: insights into BDNF/TrkB and PKC signaling. *Cells*. 2019;8:1578.

How to cite this article: Cilleros-Mañé V, Just-Borràs L, Polishchuk A, et al. M_1 and M_2 mAChRs activate PDK1 and regulate PKC βI and ε and the exocytotic apparatus at the NMJ. *The FASEB Journal*. 2021;35:e21724. https://doi.org/10.1096/fj.202002213R