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COUPLING OF PRESYNAPTIC MUSCARINIC AUTORECEPTORS TO SERINE KINASES IN LOW AND HIGH RELEASE CONDITIONS ON THE RAT MOTOR NERVE TERMINAL

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Abstract—We used intracellular recording to investigate how muscarinic acetylcholine receptors and the serine kinase signal transduction cascade are involved in regulating transmitter release in the neuromuscular synapses of the levator auris longus muscle from adult rats.

Experiments with M1 and M2 selective blockers show that these subtypes of muscarinic receptors were involved in enhancing and inhibiting acetylcholine (ACh) release, respectively. Because the unselective muscarinic blocker atropine considerably increased release, the overall presynaptic muscarinic mechanism seemed to moderate ACh secretion in normal conditions. This muscarinic function did not change when more ACh was released (high external Ca^{2+}) or when there was more ACh in the cleft (fasciculin II). However, when release was low (high external Mg^{2+} or low external Ca^{2+}) or when there was less ACh in the cleft (when acetylcholinesterase was added, AChE), the response of M1 and M2 receptors to endogenously released ACh shifted to optimize release, thus producing a net potentiation of the Mg^{2+}-depressed level.

Protein kinase A (PKA) (but not protein kinase C, PKC) has a constitutive role in promoting a component of normal release because when it is inhibited with N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2 HCl, release diminishes. The imbalance of the muscarinic acetylcholine receptors (mAChRs) (with the selective block of M1 or M2) inverts the kinase function. PKC can then tonically stimulate transmitter release, whereas PKA is uncoupled.

The muscarinic function can be explained by an increased M1-mediated PKC activity-dependent release and a decreased M2-mediated PKA activity-dependent release. In the presence of high external Mg^{2+} or low Ca^{2+}, or when AChE is added, both mAChRs may potentiate release through an M2-mediated PKC mechanism and an M1-mediated mechanism downstream of the PKC.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CAC, calphostin C; DMSO, dimethylsulfoxide; EPP, endplate potential; H-89, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2 HCl; LAL, levator auris longus; mAChR, muscarinic acetylcholine receptor; MEPP, miniature endplate potential; MNT, motor nerve terminals; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; Sp-8-BrcAMPs, adenosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, Rp-isomer, sodium salt; STK, serine threonine kinases; VDCC, voltage dependent calcium channel.

Key words: protein kinase C, protein kinase A, muscarinic receptors, neuromuscular junction, ACh release.

Locally at individual synapses, transmitter release is finely regulated by presynaptic metabotropic receptors of neurotrophins, transmitters and co-transmitters. Neurotransmitter release has been seen to be modulated by presynaptic muscarinic acetylcholine autoreceptors (mAChRs) in the cholinergic synapses (Caulfield, 1993; Allen, 1999; Slutsky et al., 1999; Minic et al., 2002; Santafé et al., 2003, 2004; Garcia et al., 2005). In the neuromuscular junction of the adult mouse (Minic et al., 2002) and rat (Santafé et al., 2003), presynaptic M1 and M2 subtypes of muscarinic receptors are involved in enhancing and inhibiting acetylcholine (ACh) release, respectively. In the adult rat, we found non-functional M3 and M4 muscarinic receptors (Garcia et al., 2005), though the M4 subtype was operative in certain newborn NMJ during the neonatal physiological synaptic elimination process (Santafé et al., 2004).

In different cellular systems, M1 and M3 receptors preferentially couple to pertussis toxin (PTX) –insensitive G-proteins of the G q/1l family to stimulate phospholipase C (and thus protein kinase C, PKC), while M2 and M4 receptor activation couples to PTX-sensitive G-proteins of the Gi/Go family to inhibit adenylyl cyclase and protein kinase A (PKA) (Caulfield, 1993; Felder, 1995; Caulfield and Birdsall, 1998; but see Nathanson, 2000). The serine threonine kinases (STK), both PKC and PKA, have been involved in the regulation of ligand-gated ion channels (Swope et al., 1999; Nelson et al., 2003, 2005) and transmitter exocytosis (Tanaka and Nishizuka, 1994; Byrne and Kandel, 1996). In a previous study, we found that both M1 and M2 mechanisms were altered when PKC, PKA or the P/Q-type calcium channel was blocked and that the muscarinic function can be explained by an increased M1-mediated PKC activity-dependent release and a decreased M2-mediated PKA activity-dependent release (Santafé et al., 2006).

ACh is the physiological agonist of the mAChRs and the conditions of the specific activation of the autoreceptors that influence release are not yet fully understood. In the present study, the data strongly suggest that mAChR-STK signaling is functionally involved in the control of transmitter release when the ACh release or the ACh level in the synaptic cleft is changed. Specifically, we performed several experiments: with high external Ca^{2+} to increase ACh release, with a specific inhibitor of acetylcholinesterase (AChE) (fasciculin II) to increase the permanence of

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ACh in the synaptic cleft, with added AChE to reduce the permanence of ACh, and with high Mg^{2+} or low Ca^{2+} in the media to reduce release.

Our findings highlight the coordinated involvement of PKC and PKA, in the intracellular cascades downstream of the mAChR activation and emphasize the role of these molecules in the control of neuromuscular transmission.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on the levator auris longus (LAL) muscle of adult Sprague–Dawley rats (30–40 days postnatal; Criffa, Barcelona, Spain). The rats were cared for in accordance with the guidelines of the European Community's Council Directive of 24 November 1986 (86/609/EEC) and local guidelines for the humane treatment of laboratory animals. All efforts were made to minimize the number of animals used and their suffering. The animals were anesthetized with 2% tribromoethanol (0.15 ml/10 g body weight, i.p.) and killed by exsanguination while deeply anesthetized.

Electrophysiological recordings

The LAL muscle with its nerve supply was excised and dissected on a Sylgard-coated Petri dish containing normal Ringer solution (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1; $NaHCO_3$, 12; Na_2HPO_4, 1 and glucose 11, continuously bubbled with 95% O_2/5% CO_2. The preparation was then transferred to a recording chamber of 1.5 ml. Experiments were performed at room temperature (22–25 °C). The bath temperature was monitored during experiments (23.4 °C±1.7, Digital Thermometer TMP 812, Letica, Barcelona, Spain). Endplate potentials (EPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (resistance: 20–40 MΩ). Recording electrodes were connected to an amplifier (AMS02; Tektronics Inc., Beaverton, OR USA), and a distant Ag–AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as reference. The signals were digitized (DIGIDATA 1322A Interface, Axon Instruments Inc., Foster City, CA, USA), stored and computer-analyzed. The software Axoscope 9.0 (Axon Instruments Inc.) was used for data acquisition and analysis.

In previous studies (for muscarinic drugs, Santafé et al., 2003; for PKC drugs, Santafé et al., 2005; for PKA drugs, Santafé et al., 2006), standard sharp-electrode intracellular recording techniques were used to show that miniature endplate potential (MEPP) amplitudes and postsynaptic resting membrane potentials were unaffected and, therefore, that all the drugs act presynaptically. We attempted to determine a baseline concentration of the drugs used. We analyzed the dose-response relationships of all the drugs on the MEPPs from adult muscles. Our most important consideration was to discard the postsynaptic effects of the drugs and choose the highest drug concentration that did not change the size of the MEPPs.

We also performed dose–response experiments to control for the effect of the specific inhibitor of AChE fasciculin II (Karlsson et al., 1984) and high external calcium in our model. We selected 350 nM for fasciculin II because the increase it produces in MEPP amplitude is consistently high and reproducible (about 100%), it does not change the MEPP frequency and it consistently increases the half-decay time of the EPPs (approx. 200%), which indicates that ACh action persists in the synaptic cleft (see also Minic et al., 2002). We selected 5 mM of external calcium because it considerably increases the release (about 100% enhancement of the EPP amplitude), but does not affect MEPP amplitude (% variation: 4.62±7.93).

During EPP recordings, we used two procedures to prevent muscle contraction. In some experiments we raised the external Mg^{2+} concentration using a modified saline solution containing 0.7 mM Ca^{2+} and 5 mM Mg^{2+}. In other experiments the muscles were cut on either side of the main i.m. nerve branch (Hubbard and Wilson, 1973). A washing out was performed with 100 ml of normal Ringer for 60 min after the muscles had been cut and before the experiment was begun. Also, the Ringer in the recording chamber was completely removed twice (3 ml) between each cell recording. Moreover, the muscle fiber preparations were partially depolarized, which inactivated voltage-dependent sodium channels. Muscle contractions were then almost eliminated but the synaptic transmission mechanisms were unaffected.

In all cases, after a muscle fiber had been impaled, the nerve was continuously stimulated (70 stimuli at 0.5 Hz) using two platinum electrodes that were coupled to a pulse generator linked to a stimulus isolation unit. We recorded the last 50 EPPs and used only the results from preparations that had a resting potential lower than −30 mV and which did not deviate by more than 5 mV during the experimental paradigms. The mean amplitude (VC_{EPP}) per fiber was calculated and corrected for non-linear summation (EPPs were usually more than 4 mV; McLachlan and Martin, 1981). This was calculated as:

$$VC_{EPP} = Vm_0/\{1 - [Vm_0/(Vm_i - Vm_R)]\}$$

where $Vm_0 = V_{EPP}/\{Vm_i - Vm_R)/(Vm_E - Vm_R)\}$; Vm_i is the membrane potential assuming a value of −80 mV; Vm_R is the reversion potential for ACh, assuming a value of −15mV; V_{EPP} is the mean amplitude of the EPP recorded; Vm_E is the value of the membrane potential for the muscular fiber recorded.

We studied a minimum of 15 fibers per muscle and usually a minimum of five muscles in each type of experiment. In the single-fiber experiments (time course of the effect of drugs on EPPs of the same permanently impaled fiber), the drug(s) were added to the bathing solution and EPPs were recorded as previously described every 15 min for a minimum of 60 min.

Statistical procedure

Values are expressed as means±S.E.M. Percentage change was defined as: {(Amplitude of the EPP in saline−amplitude during drug exposure)/Amplitude of the EPP in saline}×100. We used a one-way analysis of variance (ANOVA) to evaluate differences between groups and the Bonferroni test for multiple comparisons. When differences were evaluated only between two groups, we used Welch's two-tailed *t*-test (for unpaired values and with no assumption of equal variances). Differences were considered significant at $P<0.05$.

Chemicals

Drugs that modulate PKC activity. Phorbol 12-myristate 13-acetate (PMA, Sigma) was made up as a 10 mM stock solution in dimethylsulfoxide (DMSO; Tocris, Ellisville, MO, USA). The stock solution of Calphostin C (CAC; Sigma-Aldrich, St. Louis, MO, USA) was made up as a 2.5 mM in DMSO. Working solutions were PMA, 10 nM and CAC, 10 μM.

Drugs that modulate PKA activity. N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2 HCl (H-89, Calbiochem) was made up as a 5 mM stock solution in DMSO. The stock solution of adenosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, Rp-isomer, sodium salt (Sp-8-BrcAMPs, Calbiochem) was made up as a 5 mM in deionized water. Working solutions were Sp-8-BrcAMPs 10 μM and H-89 5 μM.

Muscarinic agents. Stock solutions were pirenzepine dihydrochloride 10 mM (Tocris), methoctramine tetrahydrochloride 1 mM (Sigma) and atropine 200 μM (Sigma). Working solutions