Protein Kinase A and Ca_v1 (L-Type) Channels Are Common Targets to Facilitatory Adenosine A_{2A} and Muscarinic M₁ Receptors on Rat Motoneurons

Laura Oliveira Paulo Correia-de-Sá

Laboratório de Farmacologia, Unidade Multidisciplinar de Investigação Biomédica, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal

Key Words

Neuromuscular transmission · Adenosine A_{2A} receptors · Muscarinic M₁ receptors · Protein kinase A · Protein kinase C · Ca_v1 (L-type) channels · Acetylcholine release

Abstract

At the rat motor endplate, pre-synaptic facilitatory adenosine A_{2A} and muscarinic M₁ receptors are mutually exclusive. We investigated whether these receptors share a common intracellular signalling pathway. Suppression of McN-A-343-induced M₁ facilitation of [³H]ACh release was partially recovered when CGS21680C (an A_{2A} agonist) was combined with the cyclic AMP antagonist Rp-cAMPS. Forskolin, rolipram and 8-bromo-cyclic AMP mimicked CGS21680C blockade of M₁ facilitation. Both Rp-cAMPS and nifedipine reduced augmentation of [³H]ACh release by McN-A-343 and CGS21680C. Activation of M₁ and A_{2A} receptors enhanced Ca²⁺ recruitment through nifedipine-sensitive channels. Nifedipine inhibition revealed by McN-A-343 was prevented by chelerythrine (a PKC inhibitor) and Rp-cAMPS, suggesting that Ca_v1 (L-type) channels phosphorylation by PKA and PKC is required. Rp-cAMPS inhibited [³H]ACh release in the presence of phorbol 12-myristate 13-acetate, but PKC inhibition by chelerythrine had no effect on release in the presence of 8-bromo-cyclic AMP. This suggests that the

involvement of PKA may be secondary to M₁-induced PKC activation. In conclusion, competition of M₁ and A_{2A} receptors to facilitate ACh release from motoneurons may occur by signal convergence to a common pathway involving PKA activation and Ca²⁺ influx through Ca_v1 (L-type) channels.

Copyright © 2005 S. Karger AG, Basel

Introduction

Functional evidence that adenosine controls synaptic transmission through a sophisticated interplay between its own receptors (namely adenosine inhibitory A₁ and facilitatory A_{2A} receptors) and receptors for other neurotransmitters/neuromodulators (e.g. acetylcholine (ACh), glutamate, γ -aminobutyric acid, neuropeptides) has been provided [for review, see 1]. At the rat neuromuscular junction, facilitation of ACh release may be balanced through tonic activation of pre-synaptic muscarinic M₁ and adenosine A_{2A} receptors [2]. While the muscarinic M₁-positive feedback mechanism is operative, the adenosine A_{2A}-receptor-mediated facilitation is suppressed. In contrast, the increasing tonic activation of A_{2A} receptors by adenosine generated during neuronal firing from the catabolism of released adenine nucleotides counteracts M₁ facilitation of ACh release and may be responsible by changes in the efficacy profile of musca-

rinic agonists. These findings may explain some controversies found in literature about the muscarinic regulation of ACh release from stimulated motor nerve terminals [3], although it remains to be elucidated the signalling mechanism involved in the cross-talk between facilitatory M_1 and A_{2A} receptors at the rat motor endplate.

Muscarinic M_1 receptors typically couple via subunits of $G_{q/11}$ protein family to phospholipase C leading to inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} mobilization and to protein kinase C (PKC) activation [4]. At the rat motor nerve terminals, facilitatory adenosine A_{2A} receptors are positively coupled to the adenylate cyclase/cyclic AMP transducing system [5]. It has also been shown that blockade of Ca_v1 (L-type) channels, which do not ordinarily participate in the release process [6–8], may attenuate facilitation of ACh release induced by neuronal M_1 and A_{2A} receptors [9, 10]. The activity of pre-synaptic Ca_v1 (L-type) channels may be modulated by a membrane-delimiting pathway involving G-proteins, as well as by phosphorylation caused by cytoplasmic messengers. High intracellular cyclic AMP levels enhance the open probability, reduce the rundown, and recruit newly functioning Ca_v1 (L-type) channels [11]. Likewise, PKC-induced phosphorylation may increase quantal ACh release by opening quiescent Ca_v1 (L-type) channels in frog motor nerves at resting potential [12] and in neurons projecting to the rat urinary bladder [13].

These findings prompted us to study whether the negative interplay between M_1 and A_{2A} receptors could result from competition for activation of common signalling molecules, in particular protein kinase A (PKA) and PKC which might trigger Ca^{2+} influx through quiescent Ca_v1 (L-type) channels, in order to break neurotransmitter overflow during synchronous activation of pre-synaptic facilitatory receptors. Due to the anatomical complexity of the mammalian neuromuscular junction, it is hard to perform biochemical studies without a significant contamination of skeletal muscle metabolism. Therefore, the results presented here reflect the actions of pharmacological tools capable of interfering with a given intracellular signalling pathway.

Materials and Methods

Preparation and Experimental Conditions

Rats (Wistar, 150–200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06:30–19:30 h)/dark (19:30–06:30 h) cycle, with food and water ad libitum. The animals were killed after stunning followed by exsanguinations. Animal handling and experiments fol-

lowed the guidelines of the International Council for Laboratory Animal Science. The experiments were performed on left phrenic nerve-hemidiaphragm preparations (4–6 mm width). Each muscle was superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001, at 37°C.

The procedures used for labeling the preparations and measuring evoked [3H]ACh release were as previously described [15], with minor modifications. Phrenic nerve-hemidiaphragm preparations were mounted in 3-ml capacity Perspex chambers heated to 37°C. Nerve terminals were labeled for 40 min with 1 μM [3H]choline (specific activity 2.5 $\mu Ci\ nmol^{-1}$) under electrical stimulation at a frequency of 1 Hz (0.04 ms duration, 8 mA). The phrenic nerve was stimulated with a glass-platinum suction electrode placed near the first division branch of the nerve trunk to avoid direct contact with muscle fibers. Washout of the preparations was performed for 60 min, by superfusion (15 ml min^{-1}) with Tyrode solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was evaluated by liquid scintillation spectrometry (% counting efficiency: $40 \pm 2\%$) after appropriate background subtraction using 2-ml bath samples collected automatically every 3 min. After the loading and washout periods, the preparation contained $(5,542 \pm 248) \times 10^3\ dpm\ g^{-1}$ wet weight of tissue and the resting release was $(132 \pm 12) \times 10^3\ dpm\ g^{-1}$ ($n = 8$). The fractional release was calculated to be $2.38 \pm 0.14\%$ of the radioactivity present in the tissue at the first collected sample.

[3H]ACh release was evoked by electrical stimulation of the phrenic nerve with trains of 750 supramaximal intensity pulses of 40 μs duration delivered at a frequency of 5 Hz. Two stimulation periods were used, starting respectively at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time) (see e.g. fig. 1). Electrical stimulation increased only the release of [3H]ACh in a Ca^{2+} - and tetrodotoxin-sensitive manner [10], while the output of [3H]choline remained unchanged [16]. Therefore, evoked [3H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period [cf. 15].

Drug Effects and Pharmacological Interactions

Test drugs were added 15 min before S_2 and were present up to the end of the experiments. The percentage change in the ratio between the evoked [3H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. When we evaluated changes in the effect of drugs induced by a modifier, the modifier was applied 15 min before starting sample collection and hence was present during the whole assay including S_1 and S_2 . When present during S_1 and S_2 , none of the modifiers significantly altered ($p > 0.05$) the S_2/S_1 ratio as compared to the S_2/S_1 ratio obtained in the absence of the modifiers (0.81 ± 0.03 , $n = 8$). None of the drugs changed significantly ($p > 0.05$) basal tritium outflow (see fig. 1, 3, 4).

Materials and Solutions

8-Bromo-adenosine-3',5'-cyclic monophosphate sodium salt (8-Br-cAMP), choline chloride, 1,9-dideoxyforskolin (1,9-ddFSK), forskolin (FSK), hemicholinium-3, (4-hydroxy-2-butyryl)-1-trimethylammonium-*m*-chlorocarbonyl chloride (McN-A-343), nifedipine, pirenzepine dihydrochloride (Sigma, St. Louis, Mo., USA); chelerythrine (CHL), 11-[2-1[(diethylamino)methyl]-1-

Fig. 1. Role of the selective muscarinic M_1 receptor antagonist, MT-7 (0.1 nM), on McN-A-343 (3 μ M)-induced facilitation of evoked [3 H]ACh release from motor nerve terminals. Shown is the time course of tritium outflow from phrenic nerve terminals taken from typical experiments in the absence (Control, open circles) and in the presence of McN-A-343 (3 μ M), used either (**a**) alone or (**b**) after pretreatment with MT-7 (0.1 nM). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present

in the tissue at the beginning of the collection period and was measured in samples collected every 3 min. [3 H]ACh release was elicited by stimulating the phrenic nerve trunk with 750 pulses delivered with a frequency of 5 Hz at the indicated times (S_1 and S_2). McN-A-343 (3 μ M) was applied 15 min before S_2 ; MT-7 (0.1 nM) was present throughout the assay including S_1 and S_2 . None of the drugs changed spontaneous tritium outflow.

piperidiny]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b] [1,4]benzodiazepine-6-one (AF-DX 116), phorbol 12-myristate 13-acetate (PMA), 4-(2-[7-amino-2-(2-furyl)(1,2,4)-triazolo(2,3-a)1,3,5]triazin-5-yl-aminoethyl)phenol (ZM241385), 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone (4-RS) (rolipram, ROL) (Tocris Cookson Inc., UK); 2-(4-[2-*p*-carboxyethylphenylamino]-5'-N-ethyl-carboxamido adenosine (CGS21680C), Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Rp-cAMPS) (Research Biochemicals, Natick, Mass., USA); muscarinic toxin 7 (MT-7) (Peptide Institute Inc., Japan); [methyl- 3 H]choline chloride (ethanol solution, 80 Ci mmol $^{-1}$) (Amersham, UK); ROL (4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) was a gift from Schering AG (Berlin, Germany). All other reagents were of the highest purity available. Stock solutions of AF-DX 116, CGS21680C, PMA, ZM241385, ROL, 1,9-ddFSK were prepared in dimethylsulfoxide (DMSO), and FSK was prepared in absolute ethanol. Nifedipine was made up in a 10-mM stock solution in ethanol, which was kept protected from the light to prevent photodecomposition. Other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20°C . Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v $^{-1}$), were observed. The pH of the superfusion solution did not change following addition of the drugs at the maximum concentrations applied to the preparations.

Statistics

The data are expressed as mean $\pm$ SEM, with *n* indicating the number of experiments. Statistical analysis of data was carried out using paired or unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Dunnett's modified *t* test. *p* values at <0.05 were considered to represent significant differences.

Results

Recently we have demonstrated that the muscarinic M_1 receptor agonist, McN-A-343 (1–30 μ M), concentration-dependently increased the release of [3 H]ACh from stimulated (5 Hz frequency, 750 pulses of 0.04 ms duration) phrenic motor nerve terminals [2]. Figure 1 shows that McN-A-343 (3 μ M)-induced enhancement ($48 \pm 6\%$, $n = 11$) of [3 H]ACh release was prevented by the muscarinic toxin isolated from the venom of the green mamba (*Dendroaspis augusticeps*), MT-7 (0.1 nM), which exhibits high subtype selectivity for M_1 ($pK_B \sim 9.8$) receptors [see 4]. Under the same experimental conditions, the muscarinic M_2 receptor antagonist, AF-DX 116 (10 nM), failed

Fig. 2a, b. Agonist-induced adenosine A_{2A} receptors activation suppresses the facilitatory effect of McN-A-343 (3 μ M) on evoked [³H]ACh release from motor nerve terminals through a mechanism positively coupled to the adenylate cyclase/cyclic AMP pathway. McN-A-343 (3 μ M) was applied 15 min before S₂. CGS21680C (2 nM) with or without ZM241385 (10 nM) or Rp-cAMPS (10 μ M), FSK (3 μ M), 1,9-ddFSK (3 μ M), ROL (300 μ M), and 8-Br-cAMP (1 mM), were present throughout the assay including S₁ and S₂. The S₂/S₁ ratios obtained under these conditions were not statistically

different from the ratio obtained in control experiments (without any drug during S₁ and S₂) (dashed horizontal line, for values see Materials and Methods). The ordinates represent evoked tritium outflow expressed by S₂/S₁ ratios. Each column represents pooled data from an n number of experiments shown in parentheses. The vertical bars represent SEM. *, **p < 0.05 (one-way ANOVA followed by Dunnett's modified t test) when compared with the effect of McN-A-343 alone or in the presence of CGS21680C, respectively.

to antagonize McN-A-343 (3 μ M) facilitation (data not shown) [cf. 2].

The ability of McN-A-343 (3 μ M) to increase [³H]ACh release was blunted upon activating facilitatory adenosine A_{2A} receptors on phrenic motor nerve terminals with CGS21680C (2 nM, applied during the whole assay including S₁ and S₂) (fig. 2a). McN-A-343 (3 μ M)-induced facilitation was restored when CGS21680C (2 nM) was applied together with the selective A_{2A} receptor antagonist, ZM241385 (10 nM). Inhibition of M₁-induced facilitation by CGS21680C (2 nM) was partially recovered when the A_{2A} receptor agonist was co-applied with the cyclic AMP antagonist Rp-cAMPS (10 μ M) (fig. 2a). Furthermore, stimulation of the adenylate cyclase/cyclic AMP pathway by FSK (3 μ M, an adenylate cyclase acti-

vator), ROL (300 μ M, a non-xanthine inhibitor of phosphodiesterase) and 8-Br-cAMP (1 mM, a cell-permeant cyclic AMP analogue) mimicked the CGS21680C (2 nM) blockade (fig. 2b). The effect of FSK seems to be specific, since 1,9-ddFSK (3 μ M), which is a FSK analogue inactive on adenylate cyclase [17], did not significantly (p > 0.05) change the enhancing effect of McN-A-343 (3 μ M) on [³H]ACh release (fig. 2b). Taken together, these results indicate that occlusion of M₁ facilitation by the A_{2A} agonist requires activation of the adenylate cyclase/cyclic AMP transduction system.

Figure 3 shows that, on the other hand, McN-A-343 (3 μ M, applied during the whole assay including S₁ and S₂) suppressed the ability of CGS21680C (2 nM, 24 $\pm$ 6%, n = 5) to facilitate [³H]ACh release, indicating that

adenosine A_{2A} receptor activity is restrained when the M₁ receptor is operative. The way muscarinic M₁ receptor decreases adenosine A_{2A} facilitation may involve PKC activation. This hypothesis was considered because application of the selective PKC inhibitor CHL together with McN-A-343 (3 μ M) restored CGS21680C (2 nM) facilitation (fig. 3a). It is worth noting that CHL was applied during the whole assay including S₁ and S₂ in a concentration (5 μ M) high enough to prevent the ability of both the PKC activator, PMA (10 μ M, 27 $\pm$ 7%, n = 4), and McN-A-343 (10 μ M, 72 $\pm$ 12%, n = 4) to facilitate [³H]ACh release (fig. 3b), without significantly affecting the facilitatory action of CGS21680C (2 nM) (fig. 3a).

Whilst in the process of investigating the negative interaction between facilitatory muscarinic M₁ and adenosine A_{2A} receptors, we made the incidental observation that inhibition of PKA activity with Rp-cAMPS (10 μ M) reduced the ability of McN-A-343 (3 μ M) to increase [³H]ACh release, in parallel to the expected blockade of the PKA-mediated facilitatory effect of CGS21680C (2 nM) (fig. 4a). Figure 4a also shows that blockade of Ca_v1 (L-type) channels with nifedipine (1 μ M) mimicked Rp-cAMPS (10 μ M) suppression of McN-A-343 (3 μ M)- and CGS21680C (2 nM)-induced facilitation of evoked

[³H]ACh release. This prompted us to investigate if the facilitatory effects of M₁ and A_{2A} receptor agonists were surmountable and if blockade of a common signal target such as the Ca_v1 (L-type) channel could occlude their effects. Incubation of motor nerve terminals 15 min before S₂ with McN-A-343 (3 μ M) plus CGS21680C (2 nM) did not enhance further the release of [³H]ACh over that seen with the more active agonist, McN-A-343 (3 μ M), alone (fig. 4b, c). Thus, if both drugs were acting on separate coincidental signalling pathways, then combination of M₁ and A_{2A} receptor agonists should have increased further

Fig. 3. Muscarinic M₁ receptor activation suppresses the ability of CGS21680C to enhance [³H]ACh release from motor nerve terminals by a mechanism involving PKC activity. **a** CGS21680C (2 nM) was applied 15 min before S₂, whereas McN-A-343 (3 μ M) with or without CHL (5 μ M) was present throughout the assay including S₁ and S₂. The S₂/S₁ ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S₁ and S₂) (dashed horizontal line, for values see Materials and Methods). The ordinates represent evoked tritium outflow expressed by S₂/S₁ ratios. Each column represents pooled data from an n number of experiments shown in parentheses. The vertical bars represent SEM. *p < 0.05 (one-way ANOVA followed by Dunnett's modified t test) when compared with the effect of CGS21680C alone. **b** Shown is the time course of tritium outflow from phrenic nerve terminals taken from typical experiments in the absence (Control, open circles) and in the presence of McN-A-343 (10 μ M), used either alone (closed circles) or after pretreatment with CHL (5 μ M, closed squares). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 3 min. [³H]ACh release was elicited by stimulating the phrenic nerve trunk with 750 pulses delivered with a frequency of 5 Hz at the indicated times (S₁ and S₂). McN-A-343 (3 μ M) was applied 15 min before S₂, whereas CHL (5 μ M) was present throughout the assay including S₁ and S₂.

[^3H]ACh release. A possible antagonistic interaction between the two receptors can also be ruled out, since maximum facilitation reached in the presence of both agonists was equivalent to that seen with the more effective compound. Moreover, nifedipine ($1\text{ }\mu\text{M}$, applied during the whole assay including S_1 and S_2) abolished the facilitatory effect caused by synchronous application of McN-A-343 ($3\text{ }\mu\text{M}$) plus CGS21680C (2 nM) before S_2 (fig. 4c).

Because Ca_v1 (L-type) channels do not ordinarily participate in the release process [6–8], we investigated if Ca^{2+} influx through these channels could be unmasked upon activating facilitatory muscarinic M_1 and adenosine $\text{A}_{2\text{A}}$ receptors [see e.g. 9, 10]. Nifedipine, used in a concentration ($1\text{ }\mu\text{M}$) considered specific for blocking Ca_v1 (L-type) channels [18], inhibited the release of [^3H]ACh only in the presence of McN-A-343 ($3\text{ }\mu\text{M}$) and/or CGS21680C (2 nM), having no significant effect under non-facilitatory conditions (fig. 5). The inhibitory action of nifedipine ($1\text{ }\mu\text{M}$) resulting from co-application of McN-A-343 ($3\text{ }\mu\text{M}$) plus CGS21680C (2 nM) had a similar magnitude to that observed when each drug was used alone (fig. 5). Thus, mutual exclusion of muscarinic M_1 and adenosine $\text{A}_{2\text{A}}$ receptors is in keeping with the hy-

Fig. 4. Activation of facilitatory muscarinic M_1 and adenosine $\text{A}_{2\text{A}}$ receptors were both prevented by blocking PKA activity and Ca^{2+} influx through Ca_v1 (L-type) channels with Rp-cAMPS ($10\text{ }\mu\text{M}$) and nifedipine ($1\text{ }\mu\text{M}$), respectively. **a** McN-A-343 ($3\text{ }\mu\text{M}$) or CGS21680C (2 nM) were applied 15 min before S_2 ; in the experiments using Rp-cAMPS ($10\text{ }\mu\text{M}$) or nifedipine ($1\text{ }\mu\text{M}$), these drugs were present throughout the assay including S_1 and S_2 . The S_2/S_1 ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S_1 and S_2) (dashed horizontal line, for values see Materials and Methods). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from 4 to 11 experiments. The vertical bars represent SEM. * $p < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test) when compared with the facilitatory effects of McN-A-343 or CGS21680C alone. **b, c** Shown is the time course of tritium outflow from phrenic nerve terminals taken from typical experiments in the absence (Control, open circles) and in the presence of McN-A-343 ($3\text{ }\mu\text{M}$, closed circles), CGS21680C (2 nM , open squares) and McN-A-343 ($3\text{ }\mu\text{M}$) plus CGS21680C (2 nM) (closed squares), applied 15 min before S_2 . In the experiments using nifedipine ($1\text{ }\mu\text{M}$, open squares in **c**), this drug was present throughout the assay including S_1 and S_2 . Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 3 min. [^3H]ACh release was elicited by stimulating the phrenic nerve trunk with 750 pulses delivered with a frequency of 5 Hz at the indicated times (S_1 and S_2).

Fig. 5. Activation of facilitatory muscarinic M_1 and adenosine A_{2A} receptors trigger Ca^{2+} influx through nifedipine-sensitive channels: role of PKA and PKC activity. Nifedipine (1 μ M) was applied 15 min before S_2 . CGS21680C (2 nM), McN-A-343 (3 μ M), Rp-cAMPS (10 μ M), and CHL (5 μ M) were present throughout the assay including S_1 and S_2 . The S_2/S_1 ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S_1 and S_2) (dashed horizontal line, for values see Materials and Methods). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from an n number of experiments shown in parentheses. The vertical bars represent SEM. * $p < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) when compared with the effect of nifedipine alone.

pothesis that both receptors share a common signalling pathway to facilitate [3 H]ACh release, which may ultimately trigger Ca^{2+} influx through physiologically 'silent' Ca_v1 (L-type) channels.

Inhibition of PKA or PKC respectively with Rp-cAMPS (10 μ M) and CHL (5 μ M) prevented the ability of nifedipine (1 μ M) to decrease evoked [3 H]ACh release in the presence of McN-A-343 (3 μ M) (fig. 5). These results demonstrate that M_1 -induced Ca^{2+} influx through Ca_v1 (L-type) channels requires parallel activation of both PKA and PKC enzymes. Since Rp-cAMPS (10 μ M) attenuated the stimulatory effect of the PKC activator, PMA (10 μ M, $27 \pm 9\%$, $n = 4$), but CHL (5 μ M) was unable to affect the facilitatory action of the enzymatically

stable cyclic AMP analogue, 8-bromo-cyclic AMP (1 mM, $30 \pm 3\%$, $n = 4$) (fig. 6), data suggest that the involvement of PKA may be secondary to PKC activation.

Discussion

ACh, in addition to its stimulatory action on muscle fibers, also acts pre-synaptically to regulate its own release at the rat neuromuscular junction [see e.g. 19]. Neuronal nicotinic autoreceptors containing $\alpha 3\beta 2$ subunits [20] mediate a short-term positive feedback mechanism, which is terminated by rapid autodesensitization [21, 22]. Dual modulation (facilitation and inhibition) of ACh release has been demonstrated using muscarinic receptor agonists and antagonists [2, 23, 24]. Controversy on the predominant role of co-expressed muscarinic M_1 -facilitatory and M_2 -inhibitory receptors in motoneurons arose from differences in their pharmacology profile and stimulus activation mode [for review, see 3]. Consensus in data interpretation is challenged if variations on the release of transmitters and other neuromodulators (e.g. adenosine) during repetitive nerve stimuli are not taken into account [15]. The predominance of muscarinic M_1 autofacilitation coincided with the appearance of small amounts of endogenous adenosine activating inhibitory A_1 receptors, which did not interfere with the M_1 receptor function [2]. Conversely, upon increasing the amount of adenosine generated at the synapse to levels capable of activating facilitatory A_{2A} receptors, the M_1 -positive feedback mechanism is weakened and the muscarinic neuromodulatory control is shifted towards M_2 inhibition. Thus, co-expression of different subtypes of receptors mediating functional responses result in confusing pharmacological profiles, which might depend on potential interactions at the transduction system level. In the present work we have, for the first time, systematically examined the transduction mechanism involved in the cross-talk between facilitatory M_1 and A_{2A} receptors in the rat neuromuscular junction.

Interactions between G-protein-coupled receptors may occur at the level of receptors, G-proteins, effectors activation or second messengers [for review, see 14]. Most frequently, adenosine A_{2A} receptors interact with other pre-synaptic receptors by coupling to the adenylate cyclase/cyclic AMP system [1]. This pathway seemed a good candidate to the molecular mechanism responsible for the occlusion of the M_1 receptor activity. Our findings strongly support this hypothesis, as pre-treatment with the cyclic AMP antagonist, Rp-cAMPS, partially reversed

the impairment of McN-A-343-induced facilitation of [^3H]ACh release in the presence of CGS21680C. Moreover, stimulators of the adenylate cyclase/cyclic AMP pathway, like FSK, ROL, and 8-Br-cAMP, mimicked the ability of CGS21680C to decrease M_1 facilitation. Although PKC has also been reported to operate cross-talk involving A_{2A} receptors in the central nervous system [25, 26], our findings do not support this possibility as the inhibition of PKC activity by CHL did not modify the facilitatory effect of CGS21680C (see fig. 3), even though CHL has been used in a concentration (5 μM) that blocked the ability of the active phorbol ester, PMA, to increase the release of [^3H]ACh. The incapacity of CHL to attenuate CGS21680C facilitation also rules out interference of the PKC inhibitor with the adenosine A_{2A} receptor [27]. Conversely, CHL prevented McN-A-343-induced facilitation of [^3H]ACh release and restored the ability of CGS21680C to enhance transmitter release in the presence of the M_1 -receptor agonist. This is in keeping with data showing that the muscarinic M_1 receptor couples via $G_{q/11}$ proteins to phospholipase C activation and PKC stimulation by diacylglycerol [4, 13].

Fig. 6. PKC-mediated facilitation of [^3H]ACh release from motor nerve terminals requires secondary activation of PKA. **a** PMA (10 μM) and 8-Br-cAMP (1 mM) were applied 15 min before S_2 . Rp-cAMPS (10 μM) and CHL (5 μM) were present throughout the assay, including S_1 and S_2 . The S_2/S_1 ratios obtained under these conditions were not statistically different from the ratio obtained in control experiments (without any drug during S_1 and S_2) (dashed horizontal line, for values see Materials and Methods). The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from 4 to 5 experiments. The vertical bars represent SEM. * $p < 0.05$ (one-way ANOVA followed by Dunnett's modified t test) when compared with the effects of PMA or 8-Br-cAMP alone. **b** Shown is a schematic representation of intracellular second messengers involved in the cross-talk between facilitatory adenosine A_{2A} and muscarinic M_1 receptors on motor nerve terminals. Stimulation of A_{2A} receptors leads to activation of adenylate cyclase (AC) and production of cyclic AMP (cAMP), thereby increasing PKA activity. Activated M_1 receptors stimulate PLC to release inositol (1,4,5)trisphosphate (IP_3) and diacylglycerol (DAG), which cause the release of intracellular Ca^{2+} and activate PKC, respectively. Secondary stimulation of PKA by M_1 -induced PKC activation is thought to precede augmentation of Ca^{2+} influx through Ca_v1 (L-type) channels and facilitation of ACh release. Therefore, coupling of M_1 and A_{2A} receptors to a common pathway involving PKA activation and Ca^{2+} recruitment through Ca_v1 (L-type) channels might explain the negative interplay between these receptors to control neurotransmitter release at the rat motor endplate.

Depending on the cell type, multiple mechanisms have been suggested to explain the negative interplay between adenylate cyclase and PLC systems. Most evidence seems to suggest that the modulation of G_q -mediated responses is due to downstream activation of PKA [14]. On the one

hand, PKA has been shown to cause phosphorylation and deactivation of PLC [28, 29]. If this were the case at the rat motor endplate, inhibition of PKA by Rp-cAMPS would increase, rather than decrease, the facilitatory action of McN-A-343 (fig. 4a). On the other hand, PKA may phosphorylate IP₃ receptors, leading to an increase in their activity [30]. This seems also very unlikely, as the effect of McN-A-343 was suppressed by the A_{2A} agonist, CGS21680C, as well as by stimulators of the cyclic AMP system, FSK, ROL, and 8-Br-cAMP. Functional weakening of the adenosine A_{2A} receptor may also result from inhibition of adenylate cyclase activity operated by PKC phosphorylation when the M₁ receptor is activated. This hypothesis agrees with the general idea of PKC operating the uncoupling of receptors from the stimulation of adenylate cyclase [31], and is consistent with the restoration of CGS21680C facilitation when McN-A-343 was co-applied with CHL. The participation of PKC in the cross-talk operated by muscarinic M₁ receptors is further strengthened as active phorbol esters (e.g. PMA) mimicked the ability of McN-A-343 to decrease facilitation of ACh release caused by ligands capable of enhancing intracellular cyclic AMP, like FSK and ROL (unpubl. observations). PKC specifically inhibits adenylate cyclase type VI activity through protein phosphorylation; via this mechanism PKC causes desensitization of adenosine A_{2A} receptors in rat PC12 cells [32].

Pre-synaptic PKA and PKC activation facilitate neurotransmitter release in various regions of the nervous system [see 5, 33, 34]. Although the exact mechanism through which receptors stimulating PKC and PKA regulate transmitter release is controversial, the observation that the facilitatory actions of adenosine A_{2A} and muscarinic M₁ receptors are mutually exclusive led to the hypothesis that both signalling pathways could share a common target. This is feasible based on several common actions of PKA and PKC. One possibility includes phosphorylation of Rab3A (a small GTP-binding protein attached to synaptic vesicles) and of its associated effectors (like rabphilin), which have been associated both with the trafficking of synaptic vesicles and with an effect on a late stage of the Ca²⁺-dependent neurotransmitter release process [35]. Given that synapses from Rab3A-knockout mice exhibit profound synaptic depression when stimulated repetitively, it is tempting to speculate that proteins of the Rab family may be a substrate for the facilitatory action of both M₁ and A_{2A} receptors at the motor endplate. Further studies using deletion mutants are needed to investigate the functional significance of Rab phosphorylation in the cross-talk between M₁ and A_{2A} recep-

tors. Another possibility is a dramatic increase in Ca²⁺ influx into nerve terminals through phosphorylated Ca_v1 (L-type) voltage-gated channels yielding to the enhancement of transmitter release [11–13]. We addressed this question by testing the effects resulting from co-application of McN-A-343 and CGS21680C after pretreatment with the Ca_v1 (L-type) channel blocker, nifedipine. Prior application of nifedipine suppressed facilitation of ACh release due to co-activation of muscarinic M₁ and adenosine A_{2A} receptors, confirming our suspicion that parallel PKC and PKA stimulations may ultimately converge to Ca²⁺ influx via facilitatory Ca_v1 (L-type) channels. Moreover, data also suggest that McN-A-343 may enhance Ca²⁺ influx via Ca_v1 (L-type) channels [9, 13] in a similar manner to that caused upon incubating the rat motor endplate with CGS21680C [10]. Our findings are consistent with knockout studies using mouse models demonstrating that M₁ receptors couple to a diffusible second messenger pathway to interact with the activity of Ca_v1 (L-type) channels [36].

Another major strength of the present work is the demonstration that the mechanism by which muscarinic M₁ receptor mobilizes Ca²⁺ through ‘quiescent’ Ca_v1 (L-type) channels requires concomitant activation of both PKC and PKA enzymes, whereas the A_{2A}-receptor-mediated action occurs through stimulation of PKA without needing PKC activity (see above). Blockade of McN-A-343-induced enhancement of nifedipine inhibition by CHL and by Rp-cAMPS is consistent with that hypothesis. Although less frequently, activation of the cyclic AMP pathway by M₁ receptors has been documented in vertebrate preparations [37–39]. Moreover, activation of PKA seems to operate facilitation of [³H]ACh release independently of PKC activity, as CHL was unable to modify the facilitatory effect of 8-Br-cAMP. Conversely, the ability of PMA to enhance [³H]ACh release requires concomitant activation of PKA, as it was prevented by the cyclic AMP antagonist, Rp-cAMPS (see fig. 6). These results further suggest that the involvement of PKA yielding to Ca²⁺ mobilization through Ca_v1 (L-type) channels may be secondary to PKC activation by M₁ receptors. It is worth noting that cross-talk leading to sequential activation of the two-enzymatic cascades has been observed in heterologous expression systems [40, 41]. Reversible PKA phosphorylation of Ca_v1 (L-type) channels may underlie activity-dependent enhancement of neurotransmitter release [42]. While constitutive PKA phosphorylation of Ca_v1 (L-type) channels may give an alternative explanation for the observation that Rp-cAMPS inhibits PKC-induced M₁ enhancement of [³H]ACh release, this

would hardly account for the functional weakening of McN-A-343 facilitation in the presence of activators of the adenylate cyclase/cyclic AMP system, FSK, ROL, and 8-Br-cAMP [cf. 13].

In conclusion, our observations provide evidence that the negative interplay between muscarinic M_1 and adenosine A_{2A} receptors operating facilitation of [3H]ACh release from the rat motor nerve terminals might be due to signal convergence to a common pathway involving PKA activation and Ca^{2+} recruitment through 'quiescent' Ca_v1 (L-type) channels (fig. 6). This mechanism may be crucial to prevent neurotransmitter overflow during intense neuronal firing, a situation where the adenosine A_{2A} -mediated tonus becomes predominant [2, 15]. It also provides

a molecular basis to elucidate some of the inconsistencies found in the literature about the muscarinic regulation of evoked ACh from stimulated motor nerve terminals.

Acknowledgements

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) grants POCTI/FCB/36545/2000 and POCTI/CVT/43368/2001. L. Oliveira was in receipt of an FCT PhD studentship. We thank Dr. M.A. Timóteo and Dr. A. Barroso for their valuable collaboration in some experiments. We also thank Mrs. M. Helena Costa e Silva, Suzete Liça and Belmira Silva for their technical assistance.

References

- Sebastião AM, Ribeiro JA: Fine-tuning neuromodulation by adenosine. *Trends Pharmacol Sci* 2000;21:341–346.
- Oliveira L, Timóteo MA, Correia-de-Sá P: Modulation by adenosine of both muscarinic M_1 -facilitation and M_2 -inhibition of [3H]acetylcholine release from the rat motor nerve terminals. *Eur J Neurosci* 2002;15:1728–1736.
- Re L: Modulation of acetylcholine release by presynaptic muscarinic autoreceptors. *Acta Physiol Pharmacol Ther Latinoam* 1999;49:215–223.
- Caulfield MP, Birdsall NJM: International Union of Pharmacology. XVII Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 1998;50:279–290.
- Correia-de-Sá P, Ribeiro JA: Evidence that the presynaptic A_{2A} -adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase. *Naunyn Schmiedeberg Arch Pharmacol* 1994;350:514–522.
- Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP: Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci* 1988;11:431–438.
- Robitaille R, Adler EM, Charlton MP: Strategic location of calcium channels at transmitter release sites of frog neuromuscular junctions. *Neuron* 1990;5:773–779.
- Correia-de-Sá P, Timóteo MA, Ribeiro JA: Influence of stimulation on Ca^{2+} recruitment triggering [3H]acetylcholine release from the rat motor-nerve endings. *Eur J Pharmacol* 2000;406:355–362.
- Somogyi GT, Zernova G, Tanowitz M, de Groat WC: Role of L- and N-type Ca^{2+} channels in muscarinic receptor-mediated facilitation of ACh and noradrenaline release in the rat urinary bladder. *J Physiol (Lond)* 1997;499:645–654.
- Correia-de-Sá P, Timóteo MA, Ribeiro JA: A_{2A} adenosine receptor facilitation of neuromuscular transmission: influence of stimulus paradigm on calcium mobilization. *J Neurochem* 2000;74:2462–2469.
- Carbone E, Carabelli V, Cesetti T, Baldelli P, Hernández-Guijo JM, Giusta L: G-Protein- and cAMP-dependent L-channel gating modulation: a manifold system to control calcium entry in neurosecretory cells. *Pflügers Arch* 2001;442:801–813.
- Arenson MS, Evans SC: Activation of protein kinase C increases acetylcholine release from frog motor nerves by a direct action on L-type Ca^{2+} channels and apparently not by depolarisation of the terminal. *Neuroscience* 2001;104:1157–1164.
- Sculptoreanu A, Yoshimura N, de Groat WC, Somogyi GT: Protein kinase C is involved in M_1 -muscarinic receptor-mediated facilitation of L-type Ca^{2+} channels in neurons of the major pelvic ganglion of the adult male rat. *Neurochem Res* 2001;26:933–942.
- Cordeaux Y, Hill SJ: Mechanisms of cross-talk between G-protein-coupled receptors. *Neurosignals* 2002;11:45–57.
- Correia-de-Sá P, Timóteo MA, Ribeiro JA: Presynaptic A_1 inhibitory/ A_{2A} facilitatory adenosine receptor activation balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm. *J Neurophysiol* 1996;76:3910–3919.
- Wessler I, Kilbinger H: Release of [3H]acetylcholine from a modified rat phrenic nerve-hemidiaphragm preparation. *Naunyn Schmiedeberg Arch Pharmacol* 1986;334:357–364.
- Laurenza A, Sutkowski EMCh, Seamon KB: Forskolin: a specific stimulator of adenylate cyclase or a diterpene with multiple sites of action. *Trends Pharmacol Sci* 1989;10:442–447.
- Dunlap K, Luebke JJ, Turner TJ: Exocytotic Ca^{2+} channels in mammalian central nervous system. *Trends Neurosci* 1995;18:89–98.
- Bowman WC, Marshall IG, Gibb AJ, Harborne AJ: Feedback control of transmitter release at the neuromuscular junction. *Trends Pharmacol Sci* 1988;9:16–20.
- Faria M, Oliveira L, Timóteo MA, Lobo MG, Correia-de-Sá P: Blockade of neuronal facilitatory nicotinic receptors containing $\alpha\beta 2$ subunits contribute to tetanic fade in the rat isolated diaphragm. *Synapse* 2003;49:77–88.
- Wessler I, Halank M, Rasbach J, Kilbinger H: Presynaptic nicotine receptors mediating a positive feed-back on transmitter release from the rat phrenic nerve. *Naunyn Schmiedeberg Arch Pharmacol* 1986;334:365–372.
- Colquhoun D, Mathie A, Mulrine NK, Ogden DC: Studies on single acetylcholine-receptor channels in muscle endplate and sympathetic neurons; in Sellin LC, Libelius R, Thesleff S (eds): *Neuromuscular Junction*. Amsterdam, Elsevier, 1989, pp 217–234.
- Wessler I: Control of transmitter release from the motor nerve by presynaptic nicotinic and muscarinic autoreceptors. *Trends Pharmacol Sci* 1989;10:110–114.
- Santafé MM, Salon I, Garcia N, Lanuza MA, Uchitel OD, Tomás J: Modulation of ACh release by presynaptic muscarinic autoreceptors in the neuromuscular junction of the newborn and adult rat. *Eur J Neurosci* 2003;17:119–127.
- Dixon AK, Widdowson L, Richardson PJ: Desensitisation of the adenosine A_1 receptor by the A_{2A} receptor in the rat striatum. *J Neurochem* 1997;69:315–321.
- Lopes LV, Cunha RA, Ribeiro JA: Cross-talk between A_1 and A_{2A} adenosine receptors in the hippocampus and cortex of young adult and old rats. *J Neurophysiol* 1999;82:3196–3203.
- Schulte G, Fredholm BB: Diverse inhibitors of intracellular signalling act as adenosine receptor antagonists. *Cell Signal* 2002;14:109–113.

- 28 Liu M, Simon MI: Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. *Nature* 1996;382:83–87.
- 29 Rhee SG, Bae YS: Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem* 1997;272:15045–15048.
- 30 Wojcikiewicz RJ, Luo SG: Phosphorylation of inositol 1,4,5-trisphosphate receptors by cAMP-dependent protein kinase. Type I, II, and III receptors are differentially susceptible to phosphorylation and are phosphorylated in intact cells. *J Biol Chem* 1998;273:5670–5677.
- 31 Houslay MD: 'Cross-talk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur J Biochem* 1991;195:9–27.
- 32 Lai HL, Yang TH, Messing RO, Ching YH, Lin SC, Chern Y: Protein kinase C inhibits adenylate cyclase type VI activity during desensitization of the A_{2A}-adenosine receptor-mediated cAMP response. *J Biol Chem* 1997;272:4970–4977.
- 33 Parfitt KD, Madison DV: Phorbol esters enhance synaptic transmission by a presynaptic, calcium-dependent mechanism in rat hippocampus. *J Physiol (Lond)* 1993;471:245–268.
- 34 Chavez-Noriega LE, Stevens CF: Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *J Neurosci* 1994;14:310–317.
- 35 Turner KM, Burgoyne RD, Morgan A: Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends Neurosci* 1999;22:459–464.
- 36 Shapiro MS, Loose MD, Hamilton SE, Nathanson NM, Gomeza J, Wess J, Hille B: Assignment of muscarinic receptor subtypes mediating G-protein modulation of Ca²⁺ channels by using knockout mice. *Proc Natl Acad Sci USA* 1999;96:10899–10904.
- 37 Brown JH, Rietow M: Muscarinic-dopaminergic synergism on retinal cyclic AMP formation. *Brain Res* 1981;215:388–392.
- 38 Enyedi P, Fredholm BB, Lundberg JM, Ånggård A: Carbachol potentiates the cyclic AMP-stimulating effects of VIP in cat submandibular gland. *Eur J Pharmacol* 1982;79:139–143.
- 39 Olina MC, Onali P: Properties of muscarinic stimulated adenylate cyclase activity in rat olfactory bulb. *J Neurochem* 1992;58:1723–1729.
- 40 Felder CC, Kanterman RY, Ma AL, Axelrod J: A transfected m1 muscarinic acetylcholine receptors stimulates adenylate cyclase via phosphatidylinositol hydrolysis. *J Biol Chem* 1989;264:20356–20362.
- 41 Jones SV, Heilman CJ, Brann MR: Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* 1991;40:242–247.
- 42 Sculptoreanu A, Figourov A, de Groat WC: Voltage-dependent potentiation of neuronal L-type calcium channels due to state-dependent phosphorylation. *Am J Physiol* 1995;269:C725–C732.