RESEARCH PAPER

Inosine induces presynaptic inhibition of acetylcholine release by activation of A$_3$ adenosine receptors at the mouse neuromuscular junction

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BACKGROUND AND PURPOSE

The role of inosine at the mammalian neuromuscular junction (NMJ) has not been clearly defined. Moreover, inosine was classically considered to be the inactive metabolite of adenosine. Hence, we investigated the effect of inosine on spontaneous and evoked ACh release, the mechanism underlying its modulatory action and the receptor type and signal transduction pathway involved.

EXPERIMENTAL APPROACH

End-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) were recorded from the mouse phrenic-nerve diaphragm preparations using conventional intracellular electrophysiological techniques.

KEY RESULTS

Inosine (100 μM) reduced MEPP frequency and the amplitude and quantal content of EPPs; effects inhibited by the selective A$_3$ receptor antagonist MRS-1191. Immunohistochemical assays confirmed the presence of A$_3$ receptors at mammalian NMJ. The voltage-gated calcium channel (VGCC) blocker Cd^{2+}, the removal of extracellular Ca^{2+} and the L-type and P/Q-type VGCC antagonists, nitrendipine and ω-agatoxin IVA, respectively, all prevented inosine-induced inhibition. In the absence of endogenous adenosine, inosine decreased the hypertonic response. The effects of inosine on ACh release were prevented by the G$_{i/o}$ protein inhibitor N-ethylmaleimide, PKC antagonist chelerytrine and calmodulin antagonist W-7, but not by PKA antagonists, H-89 and KT-5720, or the inhibitor of CaMKII KN-62.

CONCLUSION AND IMPLICATIONS

Our results suggest that, at motor nerve terminals, inosine induces presynaptic inhibition of spontaneous and evoked ACh release by activating A$_3$ receptors through a mechanism that involves L-type and P/Q-type VGCCs and the secretory machinery downstream of calcium influx. A$_3$ receptors appear to be coupled to G$_{i/o}$ protein. PKC and calmodulin may be involved in these effects of inosine.

Abbreviations

αβ-MeADP, α,β-methyleneadenosine 5'-diphosphate; ω-CgTx, ω-conotoxin GVIA; ACh, acetylcholine; BgTx-R, α-bungarotoxin coupled to tetramethylrhodamine; CAMKII, calcium/calmodulin-dependent protein kinase II; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NEM, N-ethylmaleimide, NMJ, neuromuscular junction; VGCC, voltage-gated calcium channel; ω-Aga, ω-agatoxin IVA

Introduction

At the neuromuscular junction (NMJ), it was demonstrated that adenine nucleotides as well as adenosine play an important role as modulators of transmitter release by activating different presynaptic ATP receptors and adenosine receptors respectively (reviewed by Burnstock, 2007).

It is known that ATP is co-released with acetylcholine (ACh) (Silinsky, 1975), and once in the synaptic cleft it is degraded to ADP, AMP and adenosine by a family of ecto-ATP/ADPases and ecto-5'-nucleotidases (revised by Robson *et al.*, 2006). Furthermore, AMP can also be extracellularly deaminated into the metabolite IMP bypassing adenosine formation (Cunha and Sebastião, 1991; Magalhães-Cardoso *et al.*, 2003). Both, adenosine and IMP are then metabolized to inosine; adenosine-deaminase is the enzyme that acts on the passage of adenosine to inosine (Barankiewicz and Cohen, 1985) and ecto-5'-nucleotidase is involved in the conversion of IMP to inosine (Magalhães-Cardoso *et al.*, 2003). However, purines can also be released to the synaptic space from activated muscles fibres (Smith, 1991; Santos *et al.*, 2003) and from perisynaptic Schwann cells (Liu *et al.*, 2005).

Inosine has extensive effects on many cell types; it can stimulate degranulation of mast cells (Jin *et al.*, 1997), reduce the production of pro-inflammatory cytokines TNF-α, IL-1, IL-12, and restrain the inflammatory response provoked by endotoxin (Haskó *et al.*, 2000; Liaudet *et al.*, 2002). It also inhibits the activation of human neutrophils (Marton *et al.*, 2001) and reduces ischaemia-reperfusion injury in the rat heart transplantation model (Szabó *et al.*, 2006). In the CNS, inosine was shown to enhance axon regeneration (Benowitz *et al.*, 2002; Irwin *et al.*, 2006; Zai *et al.*, 2009) and to protect neurons and astrocytes against hypoxic injury (Litsky *et al.*, 1999; Shen *et al.*, 2005; Liu *et al.*, 2006; Wu *et al.*, 2008; Ma *et al.*, 2011). At present, little is known about the role of inosine at the NMJ. Moreover, inosine was classically considered to be an inactive metabolite of adenosine since, in sartorius muscles of the frog, Ribeiro and Sebastião (1987) found that the nucleoside did not affect the amplitude of the end-plate potential (EPP). Hence, the aim of this study was (i) to investigate the effect of inosine upon spontaneous and evoked ACh release at mammalian NMJ; (ii) to determine the receptor through which inosine exerts its action; (iii) to elucidate the presynaptic mechanisms underlying the modulatory effect; (iv) to identify the signal transduction pathway involved in the inosine responses; and (v) to investigate the physiological relevance of these effects of inosine.

Methods

Preparations and solutions

Experiments were carried out on phrenic nerve-diaphragm preparations taken from 219 adult CF1 mice (30–40 g) of either sex. All animal procedures were performed under protocols approved by national guidelines, which are in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications no. 80-23) revised 1996. Mice were anaesthetized with sodium thiopental (50 mg·kg^{-1}, i.p.) and left hemidiaphragms were excised and transferred to a 5 mL chamber superfused (3 mL·min^{-1}) with Ringer Krebs solution (mM: NaCl 135, KCl 5, CaCl$_2$ 2, MgCl$_2$ 1, D-glucose 11, HEPES 5, pH 7.3–7.4, bubbled with O$_2$). In some experiments, a saline solution containing 0 CaCl$_2$, 2 mM MgCl$_2$, and 1 mM EGTA (0Ca^{2+}-EGTA) was employed in order to eliminate the inward Ca^{2+} gradient. When the KCl concentration of the Ringer Krebs solution was raised to 12–15 mM, an equal amount of NaCl was removed from the incubation medium to maintain the isotonicity. In experiments performed in 12 mM K$^+$ – 0Ca^{2+}-EGTA, 100 μM CdCl$_2$ was added to prevent Ca^{2+} outflow from depolarized nerve terminals when the electrochemical Ca^{2+} gradient was reversed. Hyperosmotic media were freshly prepared by adding 100 mM sucrose to Krebs solutions and their osmolarity were checked with a Fiske osmometer before each experiment. When using nitrendipine, experiments were performed with extreme care to minimize exposure of drug solutions to light. All recordings were carried out at room temperature (22–23°C). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Electrophysiological recordings

Miniature end-plate potentials (MEPPs) and EPPs were recorded at the end-plate region from muscle fibres using borosilicate glass microelectrodes (WP Instruments, Sarasota, FL, USA) filled with 3 M KCl, with a resistance of 5–10 MΩ. Muscle fibres with a resting membrane potential less negative than –60 mV or MEPPs/EPPs with a rise time greater than 1 ms were rejected. For EPP recordings, the phrenic nerve was stimulated supramaximally at a frequency of 0.5 Hz (0.1 ms duration) using a suction electrode attached to a stimulus isolation unit (Grass SIU5, Grass Instruments, Quincy, MA, USA) and stimulator (Grass S48). Muscle twitches were prevented by a submaximal concentration (0.8–1.6 μM) of *d*-tubocurarine. MEPP/EPP amplitudes were normalized to a resting membrane potential of – 75 mV, using the formula $V_c = [V_o × (–75)]/E$, where V_c is the corrected MEPP/EPP amplitude, V_o is the observed MEPP/EPP amplitude, and E is the resting membrane potential. Quantal content of the EPP (m) was assessed using the failure method: $m = \ln(N/n_0)$, where N is the total number of successive trials (100 at 0.5 Hz) and n_0 is the number of trials in which the response fails (absence of EPP). In this case, twitches were prevented by increasing the concentration of magnesium (MgCl$_2$ 12–14 mM) in the bathing solution.

In the experiments where the hypertonic response was evaluated, 10 junctions were previously sampled in the isotonic solution and their values averaged. In each synapse, MEPP frequency was recorded for 100 s. Then, immediately after exposure to hyperosmotic solution, synapses were sampled repeatedly from the same small area of diaphragm over brief intervals for 30 min. An effort was made to keep the intervals between sampling as short as possible. In this case, MEPP frequency was recorded for 10 s in each synapse. Tetrodotoxin 10^{-6} M was added to hypertonic solutions to prevent the muscle from twitching violently.

All signals were amplified with Axoclamp 2B (Molecular Devices, Sunnyvale, CA, USA) and digitized with Digidata

1322 (Molecular Devices) and then analysed using pClamp 8.2 software (Molecular Devices).

Data analysis

In all cases, data are reported as mean ± SEM and n represents number of animals (only left hemidiaphragm was used from each mouse for a given experiment). Areas under the hypertonic curves were calculated using Prism (version 5.01). Statistical comparisons among three or more groups were performed using one-way ANOVA followed by Tukey's or Dunnett's post-test. Two group comparisons were performed using Student's paired t-test. Differences were considered to be significant when $P < 0.05$.

Immunohistochemistry

Tissues. Diaphragm or gastrocnemius muscles were used. To obtain further insights into A_3 receptor localization, in some experiments gastrocnemius muscles were denervated by cutting out a 0.3 cm portion of the right leg sciatic nerve. For this procedure, animals were anaesthetized with ketamine 45 mg·kg^{-1}/xylazine 6 mg·kg^{-1} (injected i.p.) and, after the wound had been closed, the animals were allowed to recover for 7 days in an animal care facility under temperature- and light-controlled conditions (20–23°C, 12 h light/12 h dark cycle) with food and water provided *ad libitum*. Then, mice were anaesthetized with sodium thiopental (50 mg·kg^{-1}) and the gastrocnemius muscles were removed. Contralateral leg muscles were used as controls.

All types of muscles were fixed for 3 min in 4% paraformaldehyde in phosphate buffer (PB, 0.1 M pH 7.4) at room temperature. Then, preparations were washed in PB for 1 min, permeabilized in 1% Triton X-100 for 5 min, washed again in PB for 1 min, and finally cryoprotected in 30% sucrose in PB for no longer than 72 h. Blocks of muscle were included in a sealed plastic tube with OCT Tissue-Tek (Sakura Finetek, Inc., Torrance, CA, USA) and then frozen in isopentane precooled in dry ice.

Frozen blocks of tissue were cut transversely (8–9 μm) with a cryostat microtome, and sections were thaw-mounted onto polylysine gelatin-coated slides, air dried for 15 min and stored at –20°C.

Polyclonal antibodies and toxins. Specific primary antibodies for the A_3 receptor were purchased from (Sigma Aldrich, St Louis, MO, USA). The polyclonal antibody was produced in rabbit using a synthetic peptide corresponding to the second extracellular loop of human A_3 receptor as the immunogen. Double labelling was performed using goat anti-rabbit IgG coupled to Atto-488 (Sigma Aldrich) to identify the primary antibody and α-bungarotoxin coupled to tetramethylrhodamine (BgTx-R, Sigma Aldrich), to identify the postsynaptic ACh receptors. Antibody and toxin concentrations were as follows: anti-A_3 receptor 1:200, secondary antibody 1:200 and BgTx-R 1:2000. Antibodies were diluted in 10 mM PBS containing 3% BSA, 0.1 M L-lysine and 0.075% Triton X-100, and the BgTx-R in 10 mM PBS.

Immunofluorescence. Tissue sections were processed simultaneously for double labelling by indirect immunofluorescence and direct staining with BgTx-R. All incubations were performed at room temperature, using 10 mM PBS pH 7.4 except where stated.

Sections were permeabilized with 0.1% Triton X-100 in PBS for 5 min, rinsed in PBS for 15 min and then incubated with the primary antibody at 4°C overnight (19–20 h).

After being successively washed with PBS for 30 min, with higher ionic concentration PBS (in mM 137 NaCl, 2.7 KCl, 4.3 Na$_2$HPO$_4$, 1.4 KH$_2$PO$_4$, pH 7.2) for 30 min and with PBS for 30 min, sections were incubated simultaneously with the secondary antibody and BgTx-R for 105 min, and washed in PBS for 40 min. Finally, the sections were mounted in 1:1 10 mM PBS:glycerol. The specificity of the A_3 signal was further assessed by incubating the muscles in the absence of the primary antibody. No staining was observed in any control assays.

Microscopy and photography. Images were acquired with a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an argon/HeNe-G laser, which allows simultaneous scanning and acquisition of the immunofluorescent sections (Plan-Apochromat 100x oil-immersion objective, numerical aperture 1.4). Assessment of co-localization of A_3 receptor and ACh receptors immunoreactivity was performed using the Zeiss LSM Image Browser 4.2 software.

Chemicals

Chelerythrine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), EGTA, inosine, (*S*)-5-isoquinolinesulfonic acid 4-[2-[(5-isoquinolinylsulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl) propyl] phenyl ester 1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT-5720), α,β-methyleneadenosine 5′-diphosphate (αβ-MeADP), 3-ethyl-5 – benzyl – 2-methyl – 4 – phenylethynyl – 6 – phenyl-1, 4 – (±) – dihydropyridine – 3,5-, dicarboxylate (MRS-1191), N-ethylmaleimide (NEM), nitrendipine, 1-amino-4-[[4-[[4-chloro-6-[[3(or4)-sulfophenyl] amino]-1, 3,5-triazin-2-yl]amino]-3-sulfophenyl] amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid (reactive blue-2), 8,8′-[carbonylbis [imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (suramin), and tetrodotoxin were purchased from Sigma-Aldrich Corp.; N-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), 1-[2-chloro – [[(3-iodophenyl) methyl] amino] – 9H – purin – 9 – yl]-1- deoxy-N-methyl – β- D -ribofuranuronamide (2-Cl-IB-MECA), 2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH-58261), and N-(6-aminohexil)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were obtained from Tocris Bioscience, Ellisville, MO, USA; and ω-agatoxin IVA (ω-Aga) and ω-conotoxin GVIA (ω-CgTx) were from Alomone Labs Ltd, Jerusalem, Israel. All other reagents were of the highest purity available. Aqueous dilutions of the stock solutions were made daily, and appropriate solvent controls were done.

The drug and molecular target nomenclature conform to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Results

Effect of inosine on spontaneous and evoked acetylcholine release

The results depicted in Figure 1A show that inosine induced a dose-related decrease in spontaneous ACh release (EC_{50} 48.59 μM). The maximal decrease in MEPP frequency was obtained in the presence of 100 μM inosine ($53.3 \pm 2.0\%$ of control values, $P < 0.0001$, $n = 10$, Figure 1B and C). These effects of inosine were completely reversible on washout with inosine-free medium, without any change in MEPP amplitude (control 0.94 ± 0.06 mV; after inosine 0.94 ± 0.04 mV, $n = 6$). When analysing the effect of inosine on evoked ACh release, we observed that the nucleoside decreased EPP amplitude to $64.4 \pm 2.8\%$ of control values ($P < 0.0001$, $n = 7$) and the EPP quantal content to $49.8 \pm 9.0\%$ of control values ($P < 0.05$, $n = 4$, Figure 1D, E and F). All these findings suggest a presynaptic action of the inosine.

Inosine activates A_3 adenosine receptors

Whereas the effects of adenosine are mediated by the combined action of the entire adenosine receptor family (A_1, A_{2A}, A_{2B}, A_3), no specific inosine receptor has been identified up to now. At the mammalian NMJ, presynaptic nerve terminals contain adenosine receptors as well as ATP receptors (revised by Burnstock, 2007). Thus, to investigate the receptor to which inosine binds to, we analysed its effect on spontaneous ACh release in the presence of antagonists of different purine receptors. We found that DPCPX (0.1 μM, selective A_1 receptor antagonist), SCH-58261 (50 nM, selective A_{2A} receptor antagonist), suramin (100 μM, a non-specific P2 receptor antagonist) and reactive blue-2 (5 μM, $P2Y_{4,6,11,12,13}$ receptor antagonist) did not prevent the modulatory effect of inosine (Table 1). Conversely, pretreatment of the preparations with the specific A_3 receptor antagonist MRS-1191 (5 μM, Jiang et al., 1996; Jacobson et al., 1997) prevented inosine-mediated presynaptic inhibition of MEPP frequency (MRS-1191 91.1 ± 3.6 ($n = 4$), MRS-1191 + inosine 95.5 ± 1.8, $n = 4$, Figure 2A and B). Moreover, the selective A_3 adenosine receptor agonist 2-Cl-IB-MECA (200 nM) decreased spontaneous neurotransmitter secretion to $66.6 \pm 0.9\%$ of control values ($P < 0.001$, $n = 3$); and this effect was prevented by the A_3 receptor antagonist (MRS-1191 $97.7 \pm 1.5\%$, MRS-1191 + 2-Cl-IB-MECA $102.3 \pm 2.7\%$, $n = 3$).

As illustrated in Figure 2C and D, MRS-1191 also prevented the effect of inosine on EPP amplitude (MRS-1191 $97.8 \pm 5.3\%$ of control values, MRS-1191 + inosine $97.3 \pm$
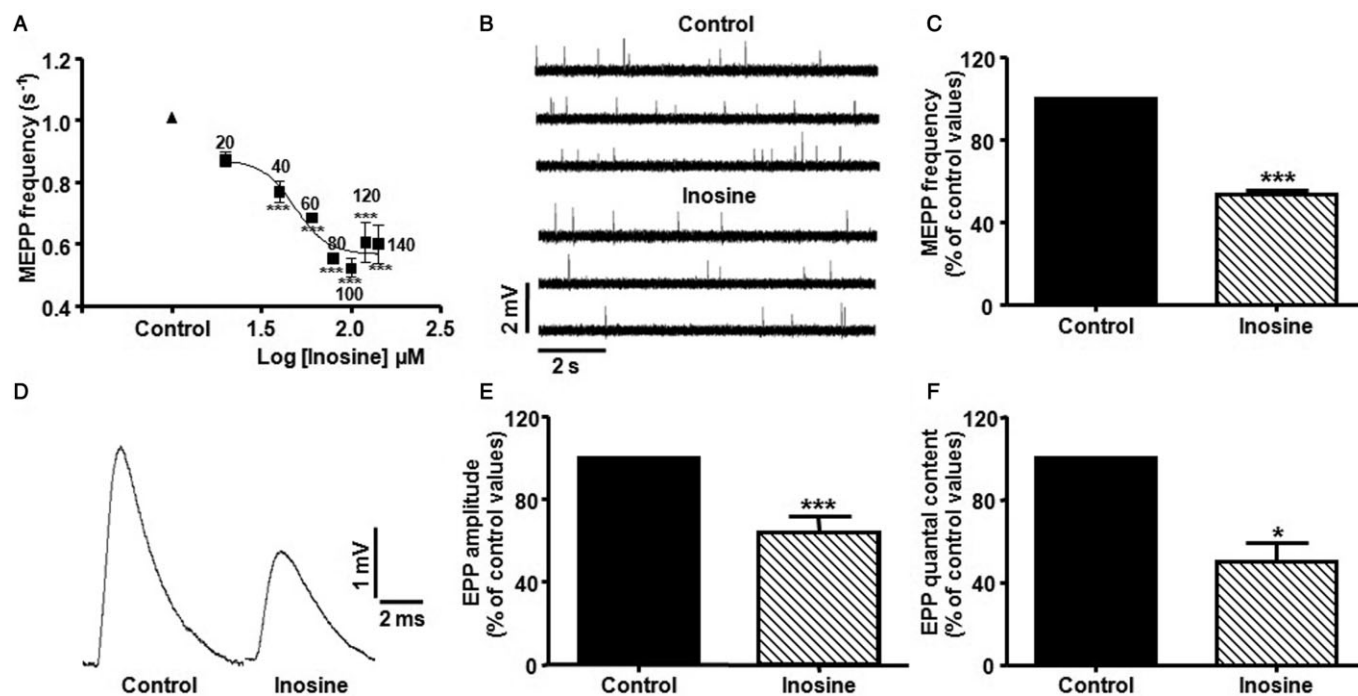

Figure 1

Inhibitory effect of inosine on spontaneous and evoked ACh release at the mouse NMJ. (A) Effect of inosine on MEPP frequency (s^{-1}) as a function of its concentration. Each point represents mean $\pm$ SEM ($n = 4$), ***$P < 0.001$ versus control, ANOVA followed by Dunnett's test; EC_{50}: 58.59 μM. (B) Representative MEPPs recorded from diaphragm muscle fibres bathed with control solution (Vm:−74.9 mV), and with 100 μM inosine (Vm:−74.2 mV). Recordings were made from the same diaphragm preparation. (C) Summary bar graph showing the presynaptic inhibitory effect of 100 μM inosine on MEPP frequency ($n = 10$). Data (mean $\pm$ SEM) are expressed as percentage of control values. ***$P < 0.0001$, Student's paired t test. (D) Effect of 100 μM inosine on EPP amplitude at mammalian NMJ. Each representative tracing is the average of 30 EPPs at a stimulation frequency of 0.5 Hz recorded from diaphragm muscle fibres bathed with control solution (Vm:−72.1 mV), and with 100 μM inosine (Vm:−73.3 mV). Recordings were made from the same diaphragm preparation. (E,F) Summary bar graphs show the presynaptic inhibitory effect of 100 μM inosine on EPP amplitude ($n = 7$) and on EPP quantal content ($n = 4$), respectively. Data (mean $\pm$ SEM) are expressed as percentage of control values. ***$P < 0.0001$, *$P < 0.05$, Student's paired t test.

Table 1

Effect of A_1, A_{2A} and P2Y receptor antagonists on the inosine-mediated modulation of spontaneous ACh secretion

Solution	MEPP frequency (% of control values)
DPCPX	99.7 ± 3.8 ($n = 4$)
DPCPX + inosine	57.8 ± 1.0 ($n = 4$)***
SCH-58261	102.5 ± 1.6 ($n = 4$)
SCH-58261 + inosine	65.8 ± 2.2 ($n = 4$)***
Suramin	99.6 ± 3.9 ($n = 4$)
Suramin + inosine	61.7 ± 3.4 ($n = 4$)***
Reactive blue-2	100.6 ± 3.3 ($n = 4$)
Reactive blue-2 + inosine	65.0 ± 1.9 ($n = 4$)***

***$P < 0.001$ versus control values and the antagonist without inosine. ANOVA followed by Tukey's test.

0.5%, $n = 4$), indicating that the modulatory action of inosine is obtained when presynaptic A_3 receptors are activated.

To assess the specific distribution of A_3 receptors at the NMJ, immunohistochemical studies were performed. Muscle cross-sections were dual-labelled with BgTX-R to identify postsynaptic ACh receptors at the motor end-plate region and, antibodies to A_3 receptors followed by staining with goat anti-rabbit IgG conjugated with Atto-488 to visualize the location of A_3 receptors. Figure 3 illustrates the co-staining of the diaphragm (A-C) and gastrocnemius (D-F) NMJs by BgTx-R and anti-A_3 antibody. To show that anti-A_3 antibodies bind to epitopes localized at the presynaptic membrane, immunostaining was performed in denervated gastrocnemius muscles (Figure 3G–I). In this case, BgTx-R labelled ACh receptors, whereas no labelling was observed with anti-A_3 antibodies. The disappearance of A_3 receptors in these sections is consistent with the degeneration of nerve terminals in response to denervation (Miledi and Slater, 1970). These results suggest that A_3 receptors are present at the presynaptic membrane of motor nerve terminals.

Presynaptic mechanisms involved in inosine-mediated modulation of transmitter release

The next aim was to elucidate the mechanisms by which inosine decreases neurotransmitter release. One possibility was that activation of A_3 receptors leads to a reduction in Ca^{2+} influx through the voltage-gated calcium channels (VGCCs) present at the presynaptic membrane of motor nerve terminals (P/Q-type, L-type and N-type VGCCs). Therefore, we first investigated the action of inosine on spontaneous ACh release in diaphragms previously incubated with the universal VGCC blocker Cd^{2+} (100 μM). As shown in Figure 4A, Cd^{2+} reduced MEPP frequency to $50.4 \pm 2.6\%$ of control values ($P < 0.001$, $n = 4$) and the addition of inosine to the bath solution did not induce any further reduction in MEPP frequency ($57.4 \pm 1.9\%$ of control values). Since L-type and N-type VGCCs are involved in tonic secretion at the mammalian NMJ, (Losavio and Muchnik, 1997), we studied the
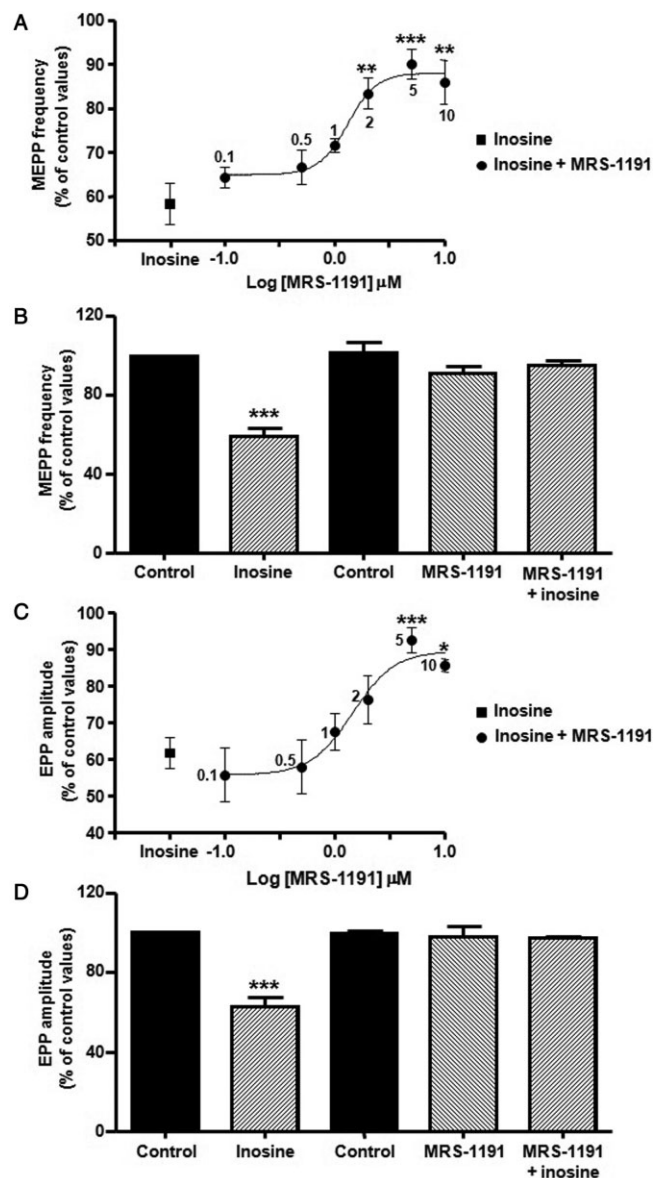

Figure 2

Inosine reduces ACh release when activating A_3 adenosine receptors. (A) Effect of increasing concentration of MRS-1191 (selective antagonist of A_3 receptors) regarding the effect of 100 μM inosine on MEPP frequency (expressed as % of control values). Each point represents mean $\pm$ SEM ($n = 3$), ***$P < 0.001$, **$P < 0.01$ versus inosine, ANOVA followed by Dunnett's test, EC_{50}: 1.31 μM. (B) MRS-1191 (5 μM) did not modify MEPP frequency, but prevented inosine effect on spontaneous secretion ($n = 4$). (C) Effect of increasing concentration of MRS-1191 on the effect of 100 μM inosine on EPP amplitude (expressed as % of control values). Each point represents mean $\pm$ SEM ($n = 3$), ***$P < 0.001$, *$P < 0.05$ versus inosine, ANOVA followed by Dunnett's test, EC_{50}: 1.45 μM. (D) MRS-1191 (5 μM) did not altered EPP amplitude, but suppressed the modulatory action of inosine on evoked ACh secretion ($n = 4$). In (B) and (D) data (mean $\pm$ SEM) are expressed as percentage of control values (black bar). ***$P < 0.001$, ANOVA followed by Tukey's test.
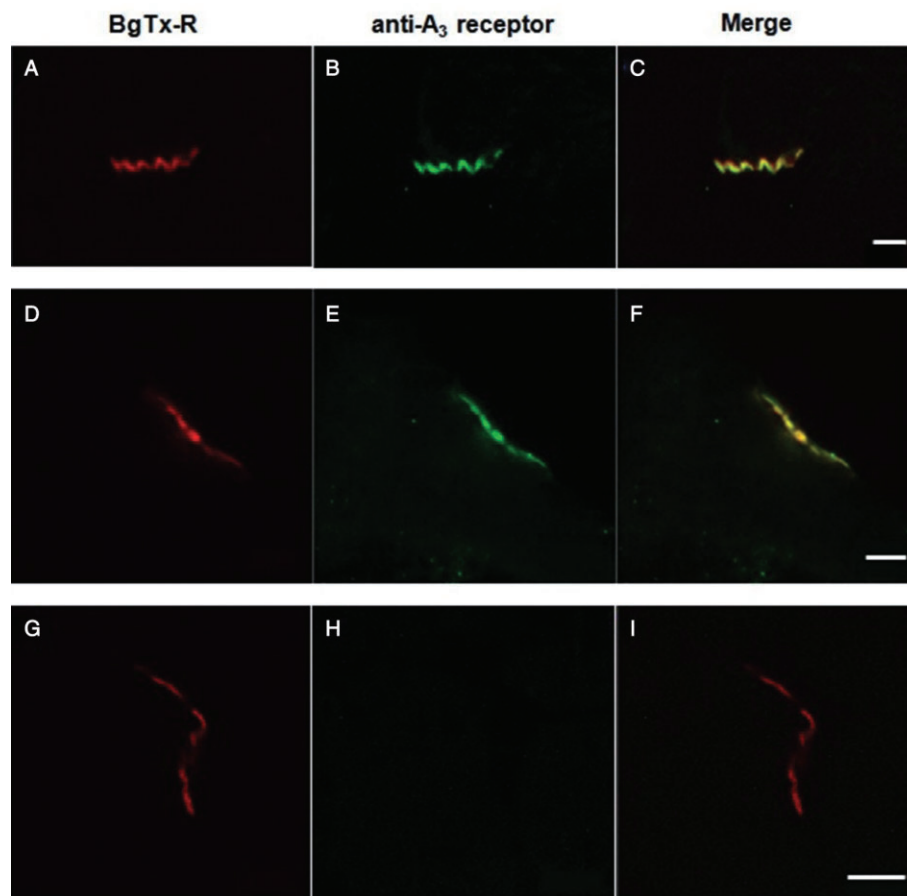
Figure 3

Distribution of A_3 receptors at the mouse NMJ in transverse sections of diaphragm (A–C) and gastrocnemius (D–I) muscles. Sections were dual-labelled with BgTx-R (red) to identify ACh receptors at the NMJ (A,D,G) and with the specific A_3 antibody, visualized with Atto-488 (green) conjugated secondary antibody (B,E,H). In innervated muscles (A–F), A_3 receptors were localized at the NMJ (B,C,E,F), whereas in denervated muscles (G–I) no labelling was observed with the A_3 antibody (H,I). Scale bar 5 μm.

effect of inosine in the presence of the specific channel blockers. The L-type VGCC blocker, nitrendipine (5 μM), reduced spontaneous secretion to $52.3 \pm 3.3\%$ of control values ($P < 0.001$, $n = 4$) and prevented inosine-induced presynaptic inhibition ($53.8 \pm 4.8\%$ of control values). On reversing the order of administration, inosine decreased MEPP frequency to $52.1 \pm 3.2\%$ of control values ($P < 0.001$, $n = 3$), and the application of nitrendipine did not induce any additional effect ($52.0 \pm 4.8\%$, Figure 4B and C). In contrast, the specific N-type VGCC blocker ω-CgTx (5 μM) reduced MEPP frequency to $65.4 \pm 3.0\%$ of control values ($P < 0.001$, $n = 4$), but the addition of inosine to the solution induced a further decrease in spontaneous ACh release ($42.9 \pm 5.4\%$ of control values, $P < 0.001$; ω-CgTx versus ω-CgTx + inosine, $P < 0.01$, Figure 4D).

The evoked release of ACh from mature mammalian motor nerve relies on Ca^{2+} entry through P/Q-type VGCCs (Protti and Uchitel, 1993). Since treatment of preparations with Cd^{2+} or the P/Q-type VGCC blocker would suppress responses induced by electrical stimulation (EPPs), we analysed the effects of inosine in preparations exposed to high K^+ concentrations, a situation in which the increase in MEPP frequency also depends on Ca^{2+} influx via P/Q-type VGCCs

(Protti and Uchitel, 1993; Losavio and Muchnik, 1997). When preparations were exposed to 15 mM K^+, MEPP frequency increased to $780.8 \pm 31.1\%$ of control values ($P < 0.001$, $n = 5$). Interestingly, the addition of inosine to preparations in 15 mM K^+ did not provoke a significant modulation of asynchronic ACh secretion ($701.2 \pm 36.1\%$ of control values, Figure 5A). To evaluate the possibility that this result is due to the extracellular accumulation of endogenous adenosine in the synaptic space occupying A_3 receptors, we studied the action of inosine at high K^+ in the presence of 100 μM αβ-MeADP (inhibitor of ecto-5'-nucleotidase, the enzyme that acts at the final step in the conversion from ATP to adenosine). In these conditions, inosine did decrease ACh secretion (15 mM K^+ + αβ-MeADP $888.5 \pm 62.3\%$ of control values, $n = 4$; 15 mM K^+ + αβ-MeADP + inosine $623.1 \pm 42.3\%$, $P < 0.05$, Figure 5B). Similarly, the inhibition of A_3 receptors with the antagonist MRS-1191 (5 μM) in 15 mM K^+ provoked an increase in asynchronous ACh release (15 mM K^+ $830.4 \pm 55.5\%$ of control values, 15 mM K^+ + MRS-1191 $1067.0 \pm 78.0\%$, $n = 4$, $P < 0.05$, Figure 5C), suggesting that, at high K^+, endogenous nucleosides modulate neurotransmitter secretion by activating A_3 receptors. Next, we decided to investi-
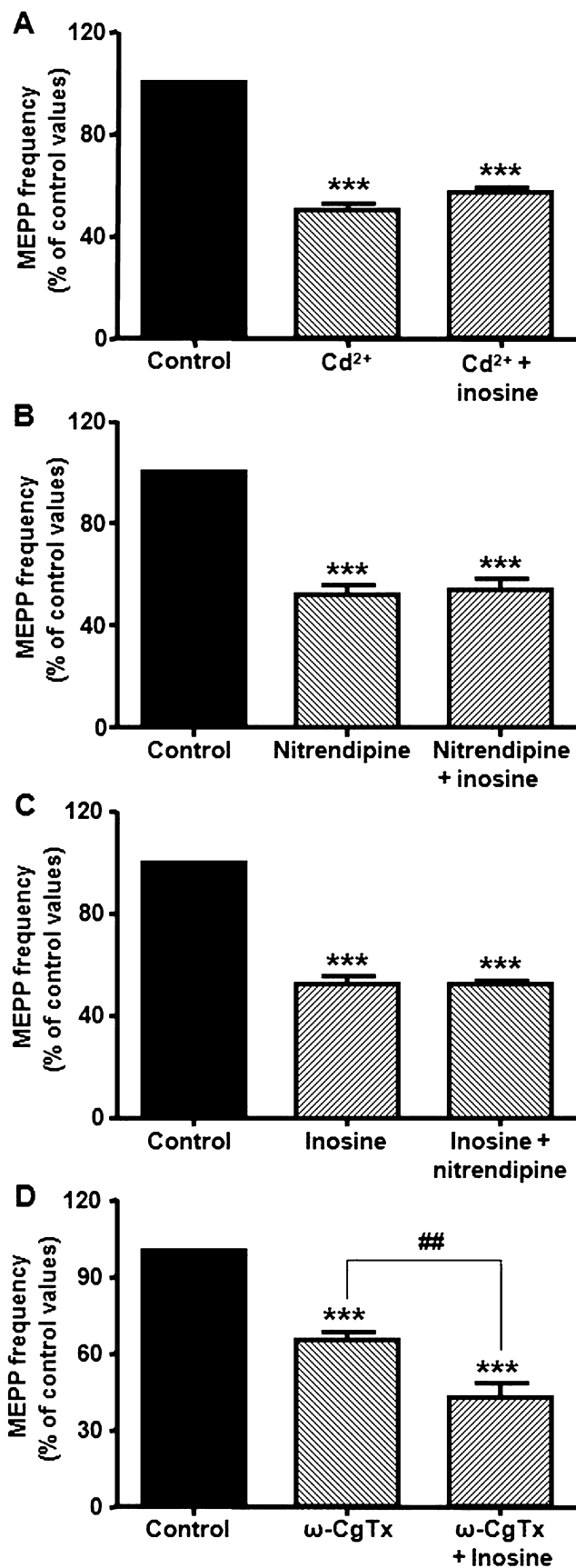
Figure 4

Effect of 100 µM inosine on VGCCs associated with spontaneous ACh secretion. (A) The universal VGCC blocker Cd^{2+} (100 µM, $n = 4$) diminished MEPP frequency and prevented the effect of inosine. (B) The L-type VGCC blocker nitrendipine (5 µM, $n = 4$) decreased spontaneous secretion and prevented the effect of inosine. (C) Nitrendipine had no effect when preparations were pre-incubated with inosine ($n = 3$). (D) The N-type VGCC blocker ω-CgTx (5 µM, $n = 4$) reduced MEPP frequency and did not prevent the modulatory effect of inosine on L-type VGCCs. Data (mean ± SEM) are expressed as percentage of control values. ***$P < 0.001$, ##$P < 0.01$, ANOVA followed by Tukey's test.

gate the action of inosine in preparations incubated with 12 mM K^+, a concentration at which the modulatory effect of inosine could be observed directly (12 mM K^+ 432.1 ± 27.3% of control values, $n = 4$; 12 mM K^+ + inosine 279.8 ± 36.7%, $P < 0.01$, Figure 6A). The non-selective VGCC blocker Cd^{2+} decreased K^+-evoked neurotransmitter secretion and prevented this presynaptic action induced by inosine (12 mM K^+ 391.6 ± 53.1% of control values, $n = 3$; 12 mM K^+ + Cd^{2+} 106.2 ± 13.2%, $P < 0.001$ vs. 12 mM K^+; 12 mM K^+ + Cd^{2+} + inosine 113.8 ± 12.0%), as shown in Figure 6B. The same behaviour was observed when extracellular Ca^{2+} was eliminated from the bathing solutions ($0Ca^{2+}$-EGTA-Cd^{2+}): 12 mM K^+ + $0Ca^{2+}$-EGTA-Cd^{2+} 43.1 ± 3.3% of control values, $n = 3$; 12 mM K^+ + $0Ca^{2+}$-EGTA-Cd^{2+} + inosine 42.7 ± 3.8% of control values (Figure 6C) or when preparations were pre-incubated with 100 nM ω-Aga, a specific P/Q-type VGCC blocker (12 mM K^+ 395.5 ± 13.5% of control values, $n = 4$; 12 mM K^+ + ω-Aga 110.5 ± 9.5%, $P < 0.001$ vs. 12 mM K^+; 12 mM K^+ + ω-Aga + inosine 108.1 ± 1.7%, Figure 6D).

Taken together, these results suggested that the activation of A_3 receptors leads to a modulation of the L-type and P/Q-type VGCCs associated with the spontaneous and evoked release of ACh respectively.

In order to investigate whether inosine-mediated inhibition is also associated with the modulation of a step in the secretory machinery down-stream of Ca^{2+} influx, we studied the effect of inosine on hypertonicity-induced enhancement of MEPP frequency, a situation that has been shown to be independent of Ca^{2+} (Furshpan, 1956; Hubbard et al., 1968; Rosenmund and Stevens, 1996; Losavio and Muchnik, 1997; Kashani et al., 2001). As shown in Figure 7A, B and C, when hypertonic solution was applied to diaphragm muscles, MEPP frequency increased from a value of 0.90 ± 0.05 s^{-1} in isotonic condition to a peak of 8.75 ± 0.62 s^{-1} ($n = 4$), and declined gradually during the continuous application of the hypertonic solution. The area under the curve was 116.9 ± 3.8 ($n = 4$). After washout with isotonic solution MEPP frequency returned to control values. The addition of inosine to the preparations reduced MEPP frequency to 0.55 ± 0.04 s^{-1} (62.1 ± 2.7% of the control responses, $p < 0.001$) in isotonic solution, but did not affect the hypertonic response, since MEPP frequency at the peak of the response was 8.13 ± 0.75 s^{-1} (93.8 ± 6.7% of the control responses) and the area under the curve was 109.0 ± 6.4 (93.7 ± 6.8% of the control responses). To rule out the possibility that endogenous adenosine, generated during the hypertonic response, may be occupying A_3
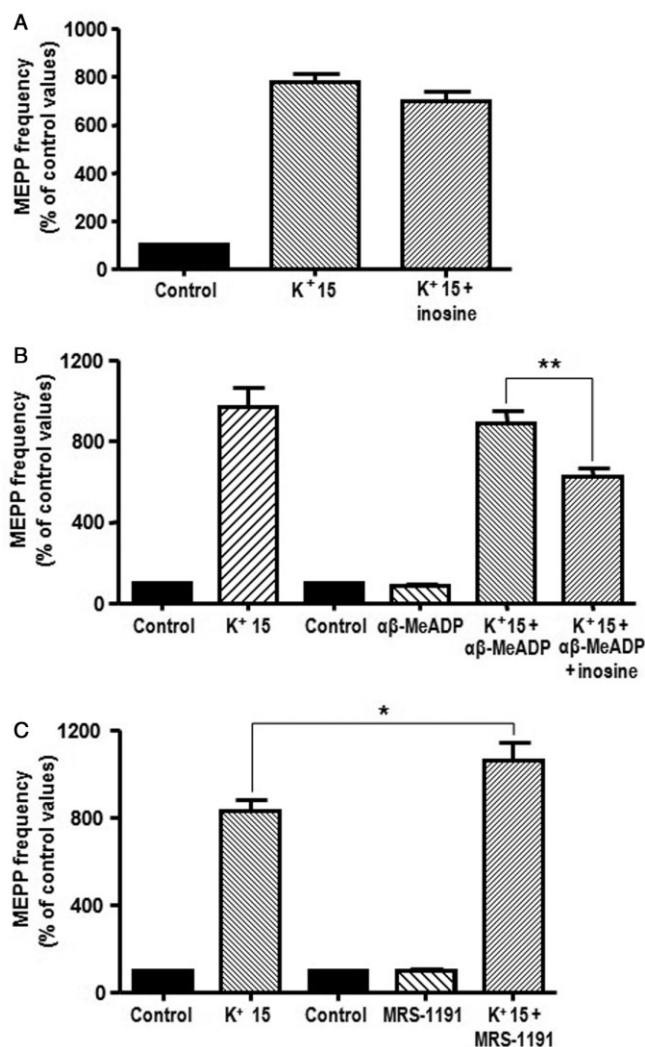
Figure 5

Activation of A_3 receptors by endogenous adenosine prevented the effect of inosine in 15 mM K^+. (A) Inosine (100 μM) failed to reduce asynchronous ACh release induced by 15 mM K^+ ($n = 5$). (B) The inhibition of the production of adenosine by 100 μM αβ-MeADP (inhibitor of ecto-5'-nucleotidase, $n = 4$) allowed the effect of 100 μM inosine on 15 mM K^+-evocked ACh release. (C) Blockade of A_3 receptors with the selective antagonist MRS-1191 (5 μM) prevented the inhibitory action of endogenous nucleosides and further increased MEPP frequency in 15 mM K^+ ($n = 4$). Data (mean ± SEM) are expressed as percentage of control values. **$P < 0.01$, *$P < 0.05$, ANOVA followed by Tukey's test.

receptors and preventing the binding of inosine, we assessed the effect of inosine in the presence of 100 μM αβ-MeADP. In this case, inosine was able to decrease the hypertonic response (peak of the response: αβ-MeADP 98.5 ± 10.7% of control response; αβ-MeADP + inosine 67.4 ± 8.9%, $P < 0.05$; area under the curve: αβ-MeADP 97.0 ± 10.4%; αβ-MeADP + inosine 64.3 ± 3.1%, $P < 0.01$, $n = 7$, Figure 7D, E, and F). This finding suggests that activation of A_3 receptors also modulates the transmitter-releasing machinery in a Ca^{2+} independent manner.

Intracellular pathways associated with the activation of A_3 adenosine receptors

It is known that A_3 receptors couple predominantly to G proteins of the $G_{i/o}$ family, leading to inhibition of adenylyl cyclase. Furthermore, activation of A_3 receptors can also stimulate PLC activity via G_q proteins (Zhou *et al.*, 1992; Ramkumar *et al.*, 1993; Abbracchio *et al.*, 1995; Palmer *et al.*, 1995). To determine whether A_3 receptors at the NMJ are coupled to $G_{i/o}$ proteins, we investigated the effect of inosine in preparations pre-incubated with NEM, a sulphydryl-alkylating reagent that interferes with $G_{i/o}$ protein-mediated second messenger pathways (Hoshino *et al.*, 1990; Shapiro *et al.*, 1994). We found that 10 μM NEM prevented the effect of inosine on MEPP frequency (NEM 125.7 ± 13.9% of control values, NEM + inosine 123.8 ± 6.6%, $n = 5$) suggesting that A_3 receptors are linked to $G_{i/o}$.

In an attempt to elucidate the transduction mechanisms associated with A_3 receptor activation, we investigated the action of inosine in the presence of inhibitors of several pathways (Table 2). The specific PKA inhibitors, H-89 (1 μM) and KT-5720 (500 nM), did not modify spontaneous ACh release or alter (mimick or block) the effect of inosine on MEPP frequency, indicating that inosine-mediated modulation was not associated with an effect on the cAMP cascade. The concentration of H-89 used in these experiments was shown to inhibit PKA in our system (De Lorenzo *et al.*, 2004; Veggetti *et al.*, 2008). When analysing the possible participation of PKC in the intracellular pathway activated by inosine, we observed that chelerythrine (5 μM), a specific inhibitor of PKC, completely prevented the inhibitory effect of inosine on spontaneous ACh secretion. Similar results were obtained when the sequence of application of drugs was reversed. These data suggest that PKC is involved in the presynaptic inhibition induced by inosine.

In our previous research, we showed that activation of A_1 receptors and P2Y receptors decreases ACh release by a Ca^{2+}-calmodulin-dependent mechanism, since this presynaptic inhibitory effect was prevented by the calmodulin antagonist W-7 (De Lorenzo *et al.*, 2004; 2006; Veggetti *et al.*, 2008). Hence, we examined the possibility that the above mechanism might be involved in the effect of inosine and found that 50 μM W-7 prevented the effects of inosine. However, pretreatment of the preparations with the specific inhibitor of calcium/calmodulin-dependent protein kinase II (CAMKII) KN-62 (10 μM) did not affect inosine's action, indicating that it is independent of the phosphorylation induced by the CAMKII.

Consistent with the above results, the effect of inosine on EPP amplitude was also blocked by 5 μM chelerythrine and 50 μM W-7, but not by 1 μM H-89 and 10 μM KN-62, suggesting that the intracellular pathways related to PKC and calmodulin are also involved with this effect of inosine (Table 2).

Discussion and conclusions

In this study, we have demonstrated that, contrary to what was classically believed, inosine is able to modulate ACh release at the mouse NMJ. We found that 100 μM inosine depressed MEPP frequency without affecting MEPP amplitude
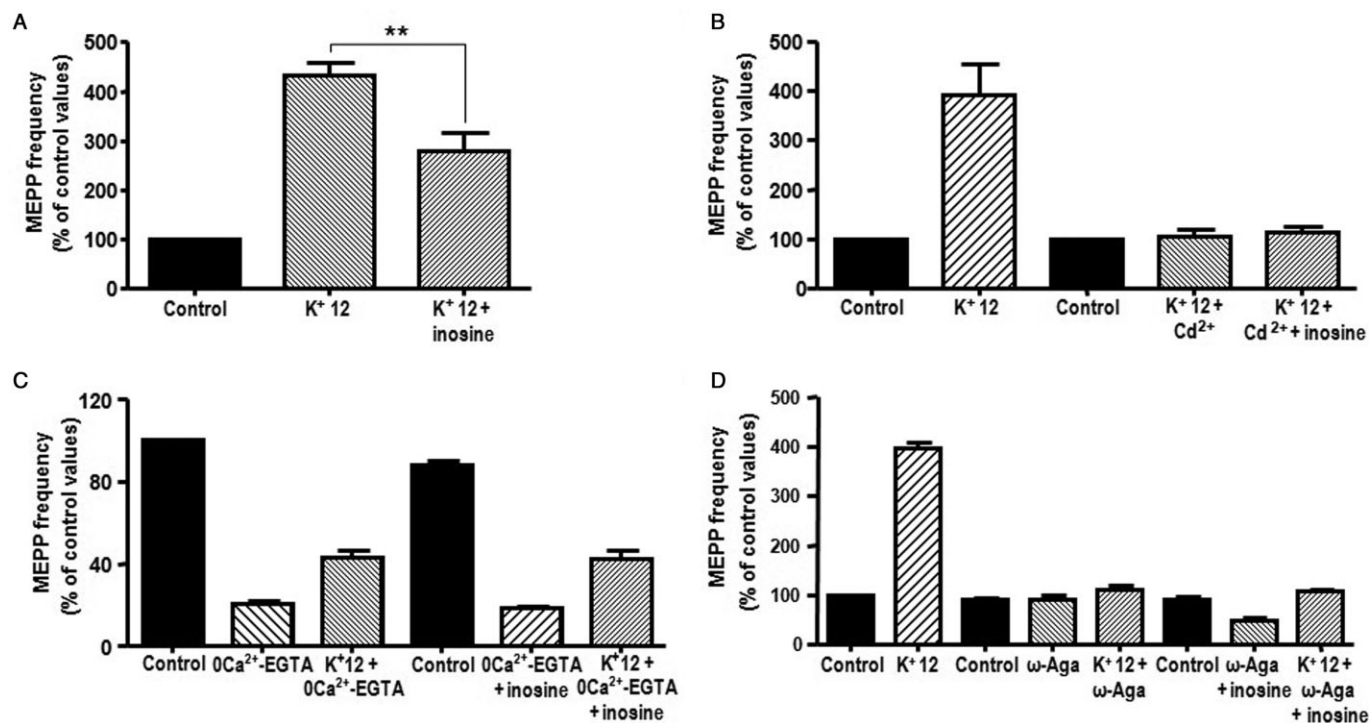
Figure 6

Inosine-mediated modulation of asynchronous ACh secretion is associated with Ca²⁺ influx through P/Q-type VGCC. (A) Inosine (100 μM) reduced asynchronous ACh release induced by 12 mM K⁺ ($n = 4$). (B,C,D) The modulatory effect of inosine on 12 mM K⁺-evoked ACh release was not observed when preparations were pre-incubated with the universal VGCC blocker Cd²⁺ (100 μM, $n = 3$), 0 Ca²⁺-EGTA ($n = 3$) or the specific P/Q-type VGCC blocker ω-Aga (100 nM, $n = 4$), respectively. In (D) it is interesting to note that 100 μM inosine decreased spontaneous ACh secretion when MEPP frequency was assessed in the presence of ω-Aga and control K⁺ (5 mM), a situation not dependent on Ca²⁺ influx through P/Q-type VGCCs ($n = 4$, $P < 0.01$). Data (mean ± SEM) are expressed as percentage of control values. **$P < 0.01$, ANOVA followed by Tukey's test.

Table 2

Effect of inhibitors of second-messenger pathways on the inosine-mediated effect

Solution values	MEPP frequency (% of control values)	EPP amplitude (% of control)
H-89	101.8 ± 5.0 ($n = 4$)	92.9 ± 0.9 ($n = 4$)
H-89 + inosine	67.9 ± 5.0 ($n = 4$)***	63.4 ± 2.4 ($n = 4$)***
KT-5720	98.4 ± 2.4 ($n = 3$)	
KT-5720 + inosine	66.4 ± 4.8 ($n = 3$)***	
Chelerythrine	102.7 ± 9.3 ($n = 4$)	102.9 ± 9.8 ($n = 4$)
Chelerythrine + inosine	105.9 ± 10.7 ($n = 4$)	109.3 ± 9.2 ($n = 4$)
Inosine	65.5 ± 5.7 ($n = 3$)***	
Inosine + chelerythrine	93.9 ± 1.5 ($n = 3$)	
W-7	97.8 ± 1.7 ($n = 4$)	107.4 ± 9.1 ($n = 4$)
W-7 + inosine	97.6 ± 7.5 ($n = 4$)	101.4 ± 8.9 ($n = 4$)
KN-62	104.4 ± 7.9 ($n = 4$)	104.3 ± 7.5 ($n = 6$)
KN-62 + inosine	68.0 ± 4.9 ($n = 4$)**	67.9 ± 8.1 ($n = 6$)**

***$P < 0.001$ and **$P < 0.01$ versus control values and the inhibitor without inosine.
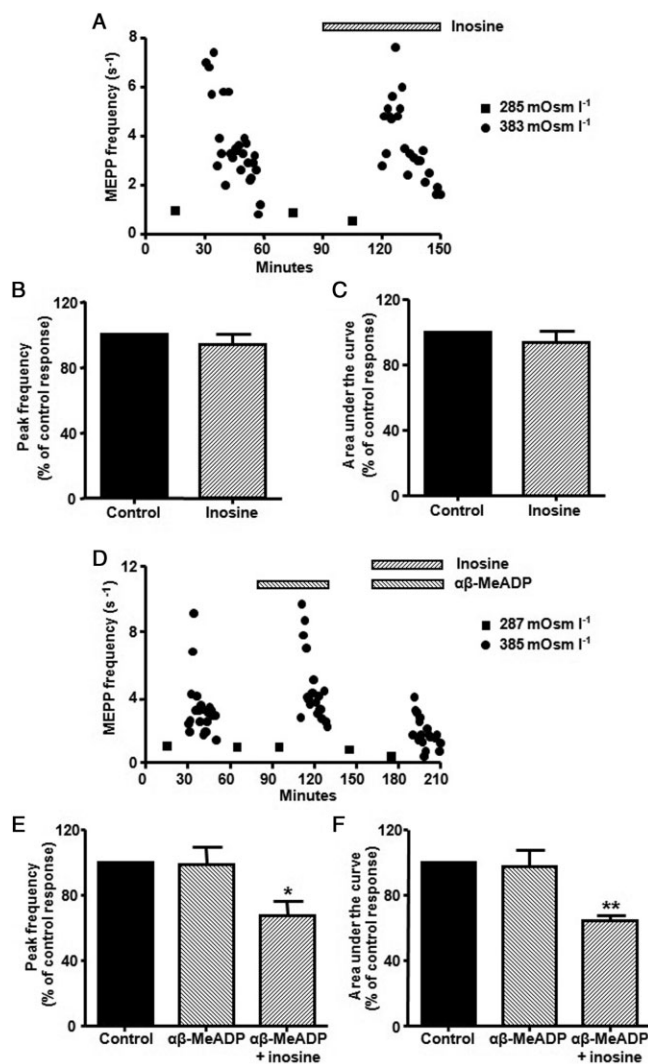ANOVA followed by Tukey's test.

Figure 7

Effect of 100 µM inosine on the hypertonic response. (A) Effect of inosine on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic conditions. (B,C) Summary bar graphs show the lack of a modulatory effect of inosine on the peak frequency and area under the curve of the hypertonic response ($n = 4$). (D) Effect of inosine on ACh release when a diaphragm muscle was exposed to isotonic and hypertonic conditions in the presence of 100 µM αβ-MeADP. (E,F) Summary bar graphs showing the modulatory effect of inosine on the peak frequency and area under the curve of the hypertonic response ($n = 7$) when the production of adenosine was inhibited by αβ-MeADP. In (A) and (D), square symbols indicate mean values from 10 synapses obtained after exposing the preparations to isotonic condition and circles represent the time course of hypertonic response (each point represents averaged value of MEPP frequency recorded from a single synapse). In (B), (C), (E), and (F), data (mean ± SEM) are expressed as percentage of control values. **$P < 0.01$, *$P < 0.05$, ANOVA followed by Tukey's test.

and reduced EPP amplitude as well as its quantum content by activating A_3 receptors. This result differs from that observed by Ribeiro and Sebastião (1987) at the frog sartorius NMJ, where they showed that 100 µM inosine had virtually no effect on EPP amplitude. These discrepancies might reflect

different sensitivities to inosine itself or a different density or distribution of A_3 receptors at the presynaptic membrane in these species.

There are no receptors known to be specific for inosine. Although the nucleoside usually binds to A_3 adenosine receptors to promote its actions (Jin *et al.*, 1997; Tilley *et al.*, 2000; Gomez and Sitkovsky, 2003), it has been shown to bind to other adenosine receptors (Gomez and Sitkovsky, 2003; Nascimento *et al.*, 2010) and even to produce its effects by a GPCR independent of adenosine receptors (Idzko *et al.*, 2004). Our results suggest that inosine binds to A_3 receptors since MRS-1191 was the only purine receptor antagonist that prevented the inosine-mediated presynaptic inhibition of spontaneous and evoked ACh secretion. The presence of A_3 receptors at motor nerve terminals was proposed by Ribeiro and Sebastião (1986), but this is the first time that the existence of these receptors has been demonstrated in pharmacological and immunohistochemical studies.

At the mammalian NMJ, activation of A_1 receptors reduces action potential-evoked ACh secretion by a mechanism that decreases P/Q-type Ca^{2+} currents (Hamilton and Smith, 1991; Silinsky, 2004) and spontaneous secretion by affecting the nitrendipine-sensitive component of MEPP frequency (De Lorenzo *et al.*, 2004). Furthermore, a Ca^{2+}-independent step in the cascade of the exocytotic process also appears to be involved in this response (Silinsky, 2005; Veggetti *et al.*, 2008). The present results provide evidence that activation of A_3 receptors interfere with calcium-dependent mechanisms, since incubation of the preparations with the universal VGCC blocker Cd^{2+} or removal of extracellular Ca^{2+} ($0Ca^{2+}$-EGTA), abolished the effect of inosine. We found that inosine reduced spontaneous ACh release by modulating L-type VGCCs, without affecting N-type VGCCs since nitrendipine prevented inosine effect, whereas in the presence of ω-CgTx, inosine induced a further reduction in MEPP frequency. Likewise, the specific antagonist of P/Q-type VGCCs, ω-Aga, abolished the modulatory action of inosine on 12 mM K^+-induced ACh release. Hence, similar to A_1 receptors, it is likely that activation of A_3 receptors at the mouse NMJ, induces presynaptic inhibition of spontaneous and evoked neurotransmitter secretion by reducing Ca^{2+} influx through L-type and P/Q-type VGCC respectively.

However, it is also possible that inosine directly modulates a step in the secretory machinery downstream of Ca^{2+} entry by an effect independent of that on VGCCs. Transmitter release can be induced by raising the tonicity of the superfusing solution, a condition known to be independent of $[Ca^{2+}]_o$ but this mechanism appears to share the major elements of the basic Ca^{2+}-triggered vesicle fusion (Dreyer *et al.*, 1987; Gansel *et al.*, 1987; Aravamudan *et al.*, 1999). Our results suggest that activation of A_3 receptors reduces ACh secretion by acting on a step downstream of Ca^{2+} entry, since inosine decreases the enhancement of neurotransmitter release induced by hypertonicity (peak and area under the curve of the hypertonic response), although this effect was only observed when the conversion from AMP to adenosine was inhibited by αβ-MeADP (see below for discussion). We have previously demonstrated that, at mammalian NMJ, hypertonic responses are not affected by the specific VGCC blockers nifedipine, ω-CgTx or ω-Aga (Losavio and Muchnik,

1997). Therefore, the decrease in the hypertonic response induced by inosine is not the result of a lower availability of intracellular Ca^{2+} provoked by the action of the nucleoside on VGCCs. As presynaptic VGCCs are intimately coupled to key elements of the synaptic vesicle docking and fusion processes (Khanna et al., 2007), it is possible that the action of an A_3 agonist on strategic components of the secretory apparatus could decrease the activation of the VGCCs. In this regard, Silinsky (2005) showed in the mouse, that cleavage of the presynaptic membrane SNARE syntaxin with botulinum toxin type C decreased the inhibitory effect of adenosine on calcium currents. Further experiments are needed to clarify whether the action of inosine on presynaptic VGCCs is associated with an effect on the secretory machinery downstream of Ca^{2+} influx or whether they are individual targets.

A_3 receptors couple primarily to proteins of the $G\alpha_i$ class and to a lesser extent to $G\alpha_{q/11}$, although additional intracellular pathways have recently been shown to be involved in receptor signalling (revised by Gessi et al., 2008). Our data showed that, at the mouse NMJ, A_3 receptors are coupled to $G_{i/o}$ protein, since incubation with NEM prevented the effect of inosine. Although the downstream mechanism of A_3 receptors is commonly based on inhibition of adenylyl cyclase resulting in a reduction in intracellular cAMP levels-transduction pathway (Zhou et al., 1992), our results indicate that this is not the primary transduction pathway by which stimulation of A_3 receptors produced its physiological effects, since the specific inhibitors of PKA, H-89 or KT-5720, neither mimicked nor occluded the effect of inosine. When evaluating the involvement of PKC, we found that the PKC inhibitor chelerythrine prevented the response to inosine. It has been suggested that $\beta\gamma$ subunits, released by G_{i-o} proteins, stimulate the PLC-diacylglycerol-PKC pathway (Dickenson and Hill, 1998; Selbie and Hill, 1998). Hence, in our experiments activation of PKC by inosine could phosphorylate presynaptic VGCCs leading to a decrease in Ca^{2+} influx, as found in cerebellar granule cells (Perroy et al., 2000) and in cardiac myocytes (Zhang et al., 1997; McHugh et al., 2000). Alternatively, PKC might phosphorylate some of the proteins involved in the exocytotic process. In particular, phosphorylation of SNAP-25 and Munc-18 by PKC has been demonstrated to reduce the affinity of these proteins with syntaxin (Fujita et al., 1996; Shimazaki et al., 1996). PKC-induced phosphorylation of SNAP-25 at Ser^{187} which modulates calcium dynamics by inhibiting VGCCs (Pozzi et al., 2008).

Our results also suggest that calmodulin is involved in the inosine-induced presynaptic inhibition, since its antagonist W-7 prevented this effect of inosine. Application of the CaMKII inhibitor KN62 did not modify the effect of inosine, demonstrating that CaMKII was not involved in this effect of calmodulin. It is known that calmodulin associates with presynaptic VGCCs including P/Q-type and L-type VGCCs (Lee et al., 1999; Dick et al., 2008). Moreover, Ivanina et al. (2000) found that at basal cellular levels of Ca^{2+}, G protein $\beta\gamma$ subunits have an inhibitory effect on L-type VGCC dependent on calmodulin. Furthermore, calmodulin can interact with proteins associated with exocytosis, for example, the GTP-bound form of Rab3 has to interact with Ca^{2+}-calmodulin in order to inhibit secretion (Coppola et al., 1999).

Another interesting finding in our study was that inosine failed to exert any modulatory effect in preparations exposed to 15 mM K^+ or on hypertonic responses (See Figures 5A and 7A–C). This lack of effect may be due to the extracellular accumulation of endogenous adenosine at the synaptic cleft, generated as result of the increased ACh secretion induced by a high K^+ concentration or hypertonicity. Indeed, we found that inhibiting the production of adenosine by addition of $\alpha\beta$-MeADP, allowed the activation of A_3 receptors by inosine and its modulatory effects. On the other hand, since inosine and adenosine access the intracellular space through the same equilibrative nucleoside transporters (Pastor-Anglada et al., 2001), it is possible that the addition of exogenous inosine might impair adenosine uptake into the cells via the equilibrative transporters increasing adenosine concentration in the synaptic cleft. Alternatively, adenosine may also be released as such from stimulated motor nerve terminals, skeletal muscle fibres and perisynaptic Schwann cell (Smith, 1991; Santos et al., 2003). In all cases, adenosine could occupy the presynaptic A_3 receptors preventing the effect of inosine. In previous studies, we demonstrated that endogenous adenosine is able to activate A_1 receptors and to modulate neurotransmitter secretion when muscles are exposed to high K^+ concentration (15 and 20 mM) or to hypertonicity (De Lorenzo et al., 2004; Veggetti et al., 2008). In the present study, tonic activation of A_3 receptors by endogenously generated adenosine was revealed when, under depolarizing conditions, the blockade of A_3 receptors by the selective antagonist MRS-1191 induced a further increase in ACh secretion, endorsing the above hypothesis.

Of the four adenosine receptors subtypes identified, A_1 and A_{2A} receptors are activated by submicromolar concentrations of adenosine (Zhou et al., 1992), whereas A_{2B} and A_3 receptors are only activated by micromolar concentrations of this nucleoside (Olah and Stiles,1995). Inosine has been found to activate rat and guinea pig A_3 receptors with K_i values in the range of 15–25 μM (Jin et al., 1997), but this nucleoside accumulates to even higher levels than adenosine in ischaemic tissues (Roth et al., 1997; Linden, 2001; Kékesi et al., 2002; Shen et al., 2005; Takahashi et al., 2010). Hence, even though A_3 receptors may not have a very high affinity for inosine, interstitial concentrations of the nucleoside may be high enough to activate these receptors in ischaemic conditions. This might be the case at the NMJ. Neurotransmitter release is affected by a deficit of oxygen (Hirsch and Gibson, 1984) and its failure produces muscular weakness during ischaemia (Eccles et al., 1966). So, an increase in the concentration of inosine in the synaptic cleft, coming from the deamination of adenosine, or from intracellular sources through equilibrative transporters, might provide a modulatory effect on ischaemic tissues, as occurs in the CNS.

In conclusion, at mammalian NMJ, inosine induces presynaptic inhibition of spontaneous and evoked ACh release by activating A_3 receptors, through a mechanism that involves L-type and P/Q-type VGCCs, and a Ca^{2+}-independent step in the cascade of the exocytotic process. We found that A_3 receptors are coupled to $G_{i/o}$ protein and that PKC and calmodulin might be involved in the action of this nucleoside. Further experiments are needed to provide information about the relative contribution of inosine to the modulatory role of purines at the NMJ, especially during hypoxia.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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