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Inhibition of spontaneous acetylcholine secretion by 2-chloroadenosine as revealed by a protein kinase inhibitor at the mouse neuromuscular junction

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1 Previous studies have reported discrepancies in the potencies of A_1 adenosine receptor agonists at mouse motor nerve terminals. In addition, conflicting results on the role of protein kinase A (PKA) in mediating the inhibitory effects of A_1 receptor agonists have been published. We thus decided to investigate the possibility of endogenous control of adenosine receptor sensitivity by protein kinases, using a variety of protein kinase inhibitors in conjunction with the adenosine receptor agonist 2-chloroadenosine (CADO).

2 CADO, at the concentration employed previously to study spontaneous ACh release in the mouse (1 μM), did not inhibit spontaneous ACh release in our experiments. However, a higher concentration of CADO (10 μM) produced highly statistically-significant reductions in spontaneous ACh release.

3 In the presence of the non-selective protein kinase inhibitor, H7 (50 μM), the potency of CADO was increased such that 1 μM CADO now reduced spontaneous quantal ACh release to approximately 63% of control.

4 Both H7, and the selective PKA inhibitor, KT5720 (500 nM) prevented increases in ACh release produced by CPT cyclic AMP (250 μM), suggesting these kinase inhibitors were blocking PKA. In contrast to H7, however, KT5720, did not reveal an inhibitory effect of 1 μM CADO. A number of other non-selective PKA inhibitors also failed to increase the potency of CADO.

5 The results suggest that an endogenous H7-sensitive process modulates the sensitivity of the mouse A_1 adenosine receptor and that the inhibitory effects of CADO are independent of cyclic AMP accumulation or PKA inhibition.

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Abbreviations: CADO, 2-chloroadenosine; CPT cyclic AMP, (8-4-(cyclopentylthio) cyclic AMP; DMPX, 3,7-dimethyl-1-propargylxanthine; H7, (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; H89, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; PKA, protein kinase A; PKC, protein kinase C; PKG, Protein Kinase G; ZM 241385, 4-(2-[7-Amino-2-(2-furyl)[1,2,4] triazolo[2,3-a] [1,3,5]triazin-5 ylamino]ethyl)phenol

Introduction

Adenosine inhibits the secretion of the neurotransmitter acetylcholine (ACh) at motor nerve endings to skeletal muscle in the rat (Ginsborg & Hirst, 1972), frog (Silinsky, 1980, 1984), and mouse (Singh *et al*., 1986; Nagano *et al*., 1992). Indeed, adenosine derived from neurally-released ATP appears to be the physiological mediator of prejunctional neuromuscular depression at low frequencies of stimulation in the frog (Redman & Silinsky, 1994) and may participate in depression in the mouse as well (Silinsky *et al*., 1999). In contrast to the results in frog and rat, however, the published literature in mouse reveals apparent differences in potencies in the ability of adenosine receptor agonists to inhibit ACh release (e.g. compare Chen *et al*., 1989 with Nagano *et al*., 1992). These results raise the possibility that the state of the mouse adenosine receptor may be controlled by endogenous factors such as the activity of protein kinases.

The role of protein kinases as mediators of the action of adenosine has been a frequent topic of investigation at motor nerve endings. In amphibia, the inhibitory effect of A_1 adenosine receptor activity is not mediated *via* cyclic AMP or its associated protein kinase A (PKA, Hirsh *et al*., 1990). In contrast to these results is the report that, in the mouse, pretreatment with the non-selective protein kinase inhibitor H7 prevented the inhibitory effects of 2-chloroadenosine (CADO) on spontaneous ACh release reflected as miniature end-plate potential (mepp) frequency (Chen *et al*., 1989). Based upon these results, it was suggested that PKA mediates the inhibitory effect of adenosine in the mouse (Chen *et al*., 1989).

It would thus appear of importance to resolve these discrepant results concerning agonist potency and the signal

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transduction mechanism by which adenosine receptors inhibit ACh release in the mouse, especially considering the availability of synaptic protein knockout strains exclusively in this species. We have thus examined the role of PKA and other protein kinases in the process by which CADO inhibits quantal ACh release at the mouse neuromuscular junction. In this study, we have restricted our experiments to spontaneous quantal ACh release to minimize the potential confounding influence of endogenous adenosine derived from the evoked release of ATP from postsynaptic sites (see e.g. Vizi *et al*., 2000). Under these conditions, the entry of Ca^{2+} through voltage activated Ca^{2+} channels is also minimized.

Methods

Electrophysiological recording of acetylcholine release in the mouse phrenic nerve-hemidiaphragm

Male Balb/c and B6129F2/J mice 20–30 g in weight were anaesthetized with 5–10 ml of diethyl ether for 3–5 min, until unresponsive to touch, and exsanguinated. The entire rib cage was excized and rinsed in physiological saline solution to remove extraneous blood. The phrenic nerve-hemidiaphragm was then isolated from the rib cage whilst it was pinned out into a Petri dish coated with Sylgard silicone, (Dow Chemical Co.), and the bathing solution was continuously gassed with a 95% 0_2 and 5% CO_2 mixture at room temperature. For electrophysiological recording, the isolated nerve-muscle preparation was pinned out onto a thin layer of Sylgard in a Perspex recording chamber. The preparation was continuously superfused with gassed physiological saline solution at room temperature (21–23°C) at a flow rate of 3.0 ml min^{-1}. Drugs were added to this superfusion solution. In all experiments, recordings were made continuously from single end-plates.

Intracellular electrodes were prepared using borosilicate glass (WPI) and filled with 3 M KCl. Electrode resistance ranged from 40–70 MΩ. Spontaneous quantal ACh release, reflected as mepp frequency, was recorded on a VCR with a digital interface (A.R. Vetter Co.), digitized using the Digidata 1200 (Axon Instruments) and analysed on a PC using the programs CDR and SCAN (Dr J. Dempster, Strathclyde Electrophysiology software suite, www.strath.ac.uk/Departments/PhysPharm/ses.htm). Alternatively, recordings were made directly onto a PC using CDR and analysed as above.

Drugs and solutions

Control physiological saline solution consisting of (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1, NaH_2HCO_3 24, dextrose 11, pH 7.2 to 7.4 when gassed with 95% 0_2 and 5% CO_2 mixture, was used in all experiments (Gage & Hubbard, 1966). H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) and DMPX (3,7-dimethyl-1-propargylxanthine were obtained from Research Biochemicals Inc. H89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride), KT5720, and staurosporine were purchased from Calbiochem Corp. ZM 241385 (4-(2-[7-Amino-2-(2-furyl)[1,2,4] triazolo[2,3-a] [1,3,5]triazin-5- ylamino]ethyl)phenol) was purchased from Tocris Cookson Inc. The remaining chemicals were purchased from the Sigma Chemical Company, including type II adenosine deaminase, E.C. no. 3.5.4.4.

Statistical methods

In order to reduce the variability associated with the sampling of multiple end-plates, all results are from experiments in which electrophysiological recordings were made continuously from single end-plates. For the individual experiments, the mean and s.e.mean of the mepp frequency per second was determined by averaging the number of mepps per s in 180 consecutive s of recording. The mean and s.e.mean for the averaged data were obtained by combining the data from the individual experiments. In all experiments, for normally distributed data (e.g. when a number of experiments performed under the same conditions were compared) a one way repeated measures analysis of variance (RM ANOVA) was used to determine whether statistically significant differences occurred between the mean values amongst the treatment groups. Bonferroni's method of pairwise multiple comparisons was utilized to isolate the groups that differed from the others. The data are expressed as mean ± 1 s.e.mean from n observations. Statistical significance was determined at the $P<0.05$ level, denoted by an asterisk (*) in the figures. Data were analysed using SigmaStat and results were plotted using SigmaPlot (SPSS, Inc.).

Results

General observations on the effects of 1 and 10 μM CADO on spontaneous ACh release

It was reported previously that 1 μM 2-chloroadenosine (CADO) reduced spontaneous ACh release (i.e., decreased mepp frequency) at the mouse neuromuscular junction (Chen *et al*., 1989). This agonist has been employed frequently to examine the effects of A_1 receptor activation because, in contrast to adenosine, it is neither a substrate for uptake nor deamination at motor nerve endings (Clarke *et al*., 1952; Phillis & Wu, 1982). Figure 1 shows that, in our experiments, spontaneous mepp frequency remained unchanged in the

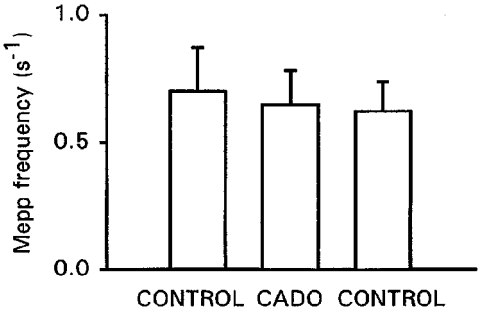

Figure 1 1 μM CADO has no effect on spontaneous ACh release in the mouse. The mean mepp frequencies ± 1 s.e.mean are shown for seven different phrenic nerve-hemidiaphragm preparations. Recordings made at steady-state are plotted for each condition, in the order shown (l–r): normal saline solution (CONTROL), 1 μM 2-chloroadenosine (CADO) and then a return to normal saline (CONTROL).

presence of 1 μM 2-chloroadenosine (CADO, $n=7$). Whilst these results disagree with some earlier results in the mouse (Singh *et al*., 1986; Chen *et al*., 1989), they are consistent with other studies where a higher IC_{50} was reported for the reduction of evoked [^{3}H]-ACh release by CADO ($\sim$9 μM; Nagano *et al*., 1992).

In contrast to these results with 1 μM CADO, statistically-significant decreases in mepp frequency were observed with 10 μM CADO. Figure 2 shows a representative individual experiment in which 10 μM reversibly decreased mepp frequency to 58% of the post-CADO control. In all 12 experiments, 10 μM CADO decreased MEPP frequency to approximately 75% of the control value.

One plausible explanation for the inability of 1 μM CADO to decrease mepp frequency, and for the range in potencies reported for adenosine analogues in the mouse is that the sensitivity of the A_1 receptor is modulated by endogenous protein kinases. In order to determine if protein kinases play a role in this system, we investigated the effects of H7, a widely-used nonselective protein kinase inhibitor (Hidaka *et al*., 1984) that has also been used in previous studies at the neuromuscular junction (see e.g. Chen *et al*., 1989; Hirsh *et al*., 1990; Sebastião & Ribeiro, 1990).

The protein kinase inhibitor H7 unmasks an inhibitory effect of CADO on spontaneous ACh release

Figure 3 illustrates a representative experiment in which the effect of 1 μM CADO was examined in the absence or presence of H7 (50 μM) during continuous recording at the same end-plate. In all 12 experiments, neither 1 μM CADO alone nor 50 μM H7 alone had any statistically-significant effect on mepp frequency ($n=12$ experiments, see also Figure 1). Previously, a similar lack of effect of H7 on ACh release was observed in the frog (Hirsh *et al*., 1990). However, in the continued presence of H7, 1 μM CADO decreased mepp frequency to 68% of the control value in the experiment of Figure 3, a highly significant decrease in spontaneous ACh release ($P<0.05$). In all seven experiments, 1 μM CADO in the presence of H7 (50 μM) decreased mepp frequency to 63.0$\pm$5.2% of Control (see Figure 4). This magnitude of reduction in mepp frequency is similar to the maximal level that has been observed previously (e.g. Singh *et al*., 1986; Chen *et al*., 1989; Nagano *et al*., 1992). Thus under our experimental conditions, H7 increased the sensitivity of the motor nerve ending to A_1 adenosine receptor agonists.

The results thus far suggest that the non-selective protein kinase inhibitor H7 unmasks the A_1 receptor-mediated

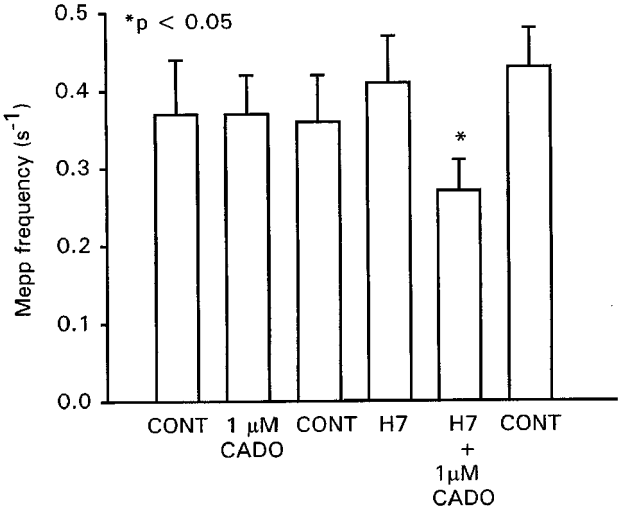

Figure 3 A representative experiment illustrating that 1 μM CADO inhibits spontaneous ACh release in the presence of H7. Recordings made at steady-state are plotted for each condition, in the order shown (l–r): normal saline control solution (CONT), 1 μM CADO, control (CONT), 50 μM H7,50 μM H7 with 1 μM CADO (H7+1 μM CADO), and control (CONT). For details of statistical analyses for the representative experiments in this study, see Methods.

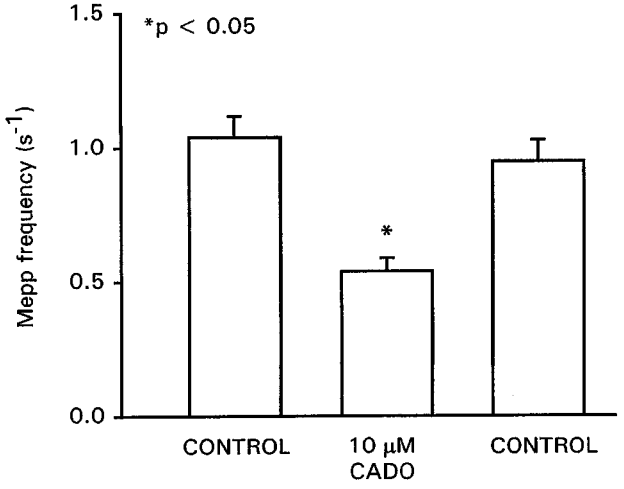

Figure 2 10 μM CADO inhibits spontaneous ACh release in the mouse. The mean mepp frequencies $\pm$1 s.e.mean are shown for a representative experiment. In these and all other experiments, mepps were monitored continuously from the same end-plate. Recordings made at steady-state are plotted for each condition, in the order shown (l–r): normal saline solution (CONTROL), 10 μM 2-chloroadenosine (CADO) and normal saline (CONTROL).

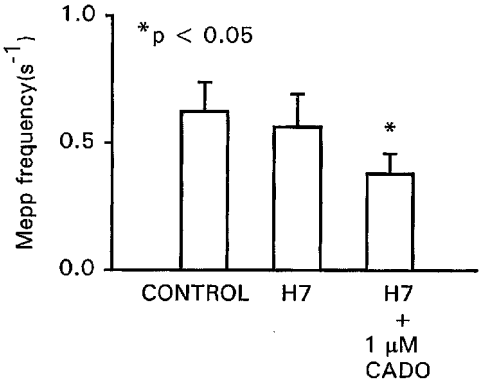

Figure 4 1 μM CADO inhibits spontaneous ACh release in the presence of H7 (averaged data). The mean mepp frequencies $\pm$1 s.e.mean are shown for seven different phrenic nerve-hemidiaphragm preparations. Recordings made at steady-state are plotted for each condition, in the order shown (l–r): normal saline solution (CONTROL), 1 μM 2-chloroadenosine (H7) and then 50 μM H7 with 1 μM CADO (H7+CADO). H7 did not significantly alter the mean mepp amplitudes ($n=12$ preparations) in these studies. Thus H7 is unlikely to exert a the type of postjunctional nicotinic receptor/ion channel blocking action in the mouse as was observed in the frog (Hirsh *et al*., 1990; Sebastião & Ribeiro, 1990).

inhibition of spontaneous ACh release by CADO. Because of the lack of selectivity of H7, we wished to determine if this effect is mediated *via* cyclic AMP and PKA.

Both H7 and the selective PKA inhibitor KT 5720 blocked the effect of a membrane permeable cyclic AMP analogue, but only H7 unmasked the inhibitory effect of CADO

In the absence of protein kinase inhibitors, the membrane permeable cyclic AMP derivative, (8-4-(cyclopentylthio) cyclic AMP (CPT cyclic AMP; see Hirsh *et al.*, 1990) increased spontaneous ACh release 1.8 fold ($n=7$ experiments – compare the first and last columns of Figures 5 and 6). H7 (50 μM, Figure 5, $n=6$ experiments) blocked the stimulatory effects of CPT cyclic AMP, suggesting that H7 is inhibiting PKA in our system. The PKA inhibitor KT5720 has been previously reported to be a highly potent and selective PKA inhibitor ($K_i \cong 56$ nM) with no significant effect on either PKC or Protein Kinase G (PKG). Indeed, as Figure 6 shows, 500 nM KT5720 blocked the stimulatory effects of CPT cyclic AMP on mepp frequency ($n=5$ experiments). KT5720 thus appears to be an appropriate PKA antagonist to determine if the effect of H7 on adenosine receptor activation is mediated through an action on PKA.

Figure 7 depicts the experimental results with KT 5720 and CADO. Note that 1 μM CADO applied in the presence of 500 nM KT5720 failed to produce a significant inhibitory effect on spontaneous ACh release (Figure 7, $n=5$ experiments). This contrasts with the effects of H7 depicted in Figures 3 and 4. Figure 7 also shows that 500 nM KT5720 did not alter the level of spontaneous ACh release in the absence of other agents.

The results thus suggest that PKA is not the protein kinase responsible for modulating the sensitivity of A_1 adenosine receptors to 1 μM CADO.

Other protein kinase inhibitors do not mimic H7

We screened several other protein kinase inhibitors which block PKA or PKC with different degrees of selectivity. Staurosporine (100 nM), another potent, cell permeable and broad spectrum PK inhibitor selective for PKA, PKC and PKG did not reproduce the effect of H7 (mepp frequency in staurosporine, 0.51 ± 0.07 s^{-1}; mepp frequency in staurosporine ± 1 μM CADO, 0.45 ± 0.06 s^{-1}; $n=6$ experiments). In addition, other more selective protein kinase inhibitors also did not mimic H7. These include the selective PKA inhibitor H89 (mepp frequency in 2 μM H89, 0.78 ± 0.17 s^{-1}; mepp frequency in 2 μM H89 + 1 μM CADO, 0.98 ± 0.20 s^{-1}, $n=5$ experiments), the specific PKA antagonist, A3 (mepp frequency in 50 μM A3, 5.67 ± 0.16 s^{-1}; mepp frequency in 50 μM A3 + 1 μM CADO, 5.64 ± 0.18 s^{-1}, $n=1$ preliminary

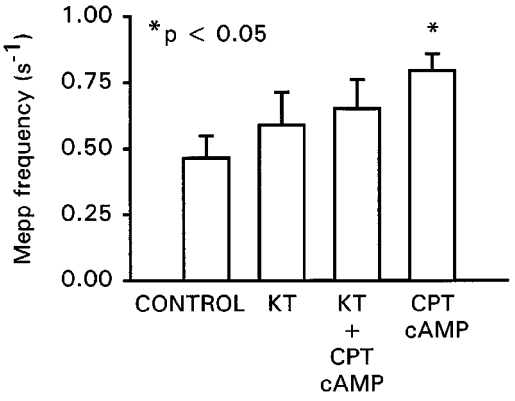

Figure 6 KT5720 does not alter spontaneous ACh release and inhibits the increase in spontaneous ACh release produced by CPT cyclic AMP. The mean mepp frequencies ± 1 s.e. mean are shown for five different phrenic nerve-hemidiaphragm preparations. Recordings made at steady-state are plotted for each condition, in the order shown (l–r):, normal saline solution (CONTROL), 500 nM KT5720 (KT), 500 nM KT5720 with 250 μM CPT cyclic AMP (KT + CPT cAMP), and then 250 μM CPT cyclic AMP alone (CPT cAMP).

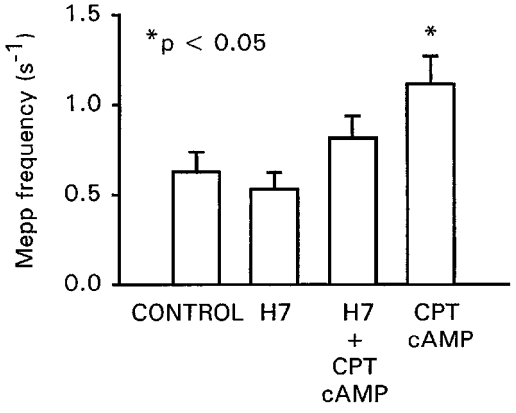

Figure 5 H7 does not alter spontaneous ACh release and inhibits the increase in spontaneous ACh release produced by CPT cyclic AMP. The mean mepp frequencies ± 1 s.e.mean are shown for five different phrenic nerve-hemidiaphragm preparations. Recordings made at steady-state are plotted for each condition, in the order shown (l–r):, normal saline solution (CONTROL), 50 μM H7 (H7), 50 μM H7 and 250 μM CPT cyclic AMP (H7 + CPT cAMP), 250 μM CPT cyclic AMP (CPT cAMP).

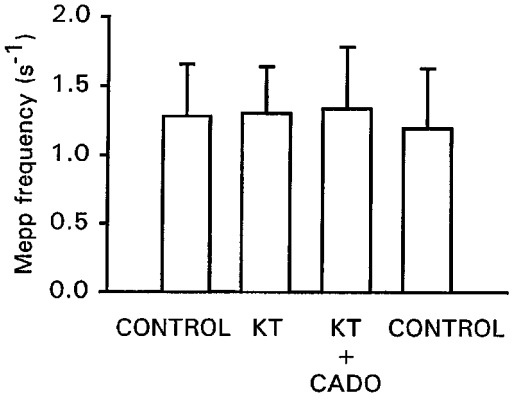

Figure 7 KT5720 does not alter the effect of CADO on spontaneous ACh release in the mouse. The mean mepp frequencies ± 1 s.e.mean are shown for five different phrenic nerve-hemidiaphragm preparations. Recordings made at steady-state are plotted for each condition, in the order shown (l–r):, normal saline solution (CONTROL), 500 nM KT5720 (KT), 500 nM KT5720 with 1 μM CADO (KT + CADO) and then normal saline solution (CONTROL).

experiment) , and the specific PKC antagonist, Bisindolylmalemide I (mepp frequency in 100 nM Bisindolylmalemide, 1.46 ± 0.09, s^{-1}; mepp frequency in 100 nM Bisindolylmalemide + 1 μM CADO: 1.33 ± 0.08 s^{-1}, $n = 1$ preliminary experiment).

The sensitivity of A_1 adenosine receptors is not affected by crosstalk with A_2 receptors or by endogenous adenosine

In contrast to the inhibitory effect of A_1 receptor activation on ACh release, A_{2a} adenosine receptors stimulate ACh release from rat motor nerve endings (Correia-De-Sá *et al*., 1994). In addition, cross talk between A_1 and A_{2a} receptors has been observed in rat brain whereby A_1 receptors are desensitized by A_{2a} receptors via the action of protein kinases (Dixon *et al*., 1997; Lopes *et al*., 1999). To investigate the possibility that A_{2a} receptor activation by endogenous adenosine mediates the reduced sensitivity to CADO, we examined the effect of the highly selective A_{2a} antagonist ZM 241385 on spontaneous ACh release and on the action of 1 μM CADO. As ZM 241385 would be expected to block tonic A_{2a} receptor activation, the failure of 1 μM CADO to inhibit ACh release in the presence of ZM 241385 would suggest that desensitization *via* endogenous A_{2a} receptor activation is not responsible for the reduced sensitivity to 1 μM CADO. Indeed, 250 nM ZM 241385 did not alter spontaneous ACh release either alone (Control mepp frequency, 0.38 ± 0.03 s^{-1}; mepp frequency in ZM 241385, 0.38 ± 0.03 s^{-1}; $n = 5$ experiments) or when co-applied with 1 μM CADO (mepp frequency in ZM 241385 + CADO, 0.37 ± 0.03 s^{-1}; $n = 5$ experiments). In additional experiments with the less selective A_2 antagonist DMPX (100 μM), a similar lack of effect was found (Control mepp frequency, 0.73 ± 0.12, s^{-1}; mepp frequency in DMPX, 0.74 ± 0.08, s^{-1}; mepp frequency in DMPX + 1 μM CADO, 0.88 ± 0.12 s^{-1}; $n = 2$ experiments). These results thus suggest that desensitization of A_1 receptors *via* A_{2a} receptor activation is not responsible for the failure of 1 μM CADO to reduce mepp frequency. Finally, 1 u ml^{-1} adenosine deaminase, which rapidly degrades any endogenous adenosine to inosine, thereby eliminating tonic adenosine receptor activation (see Sebastião & Ribeiro, 1985; Redman & Silinsky, 1994; Correia-De-Sá *et al*., 1996) also failed to alter the rate of spontaneous ACh release (Control mepp frequency, 0.87 ± 0.17 s^{-1}; mepp frequency in the presence of adenosine deaminase, 0.89 ± 0.18; $n = 5$ experiments). The results thus suggest that prior activation of adenosine receptors is not responsible for the absence of the effect of 1 μM CADO.

Discussion

In this study we report two principal results related to the role of protein kinases in the process by which A_1 adenosine receptor activation is transduced into a reduction in spontaneous ACh release. The most intriguing finding is that the sensitivity of adenosine receptors at mouse motor nerve endings is modulated by an endogenous process sensitive to the protein kinase inhibitor H7. Indeed, our surprising results in which 1 μM CADO fails to inhibit spontaneous ACh release unless H7 is present suggests that an endogenous protein kinase other than PKA, PKC or PKG modulates the sensitivity of the mouse A_1 adenosine receptor. This purine receptor may thus be desensitized, perhaps *via* phosphorylation, reducing the ability of CADO to activate the receptor. Although desensitization has not been observed for A_1 receptors on motor nerve endings in the frog (Silinsky, 1984) or the rat (Ginsborg & Hirst, 1972), it has been noted in rat brain preparations (Dixon *et al*., 1997; Lopes *et al*., 1999). This observation may indeed explain the lower efficacy of adenosine congeners on mouse motor nerve endings when compared to frog or rat. Specifically, the maximum inhibitory effect of adenosine on ACh release in the mouse is to approximately 67% of control level (our present results and Singh *et al*., 1986), in contrast to the reduction to 50% reported in the frog (Silinsky, 1984) and the rat (Ginsborg & Hirst, 1972). Adenosine analogues also have reduced potencies as inhibitors of evoked ACh release in the mouse when compared to other species (*cf*. Nagano *et al*., 1992 and Silinsky, 1984). These studies do not rule out the possibility that the effects of H7 occur independently of protein kinase inhibition, through some hitherto unknown H7 binding site.

The other result of significance is that PKA is unlikely to mediate the effects of A_1 adenosine receptor activation at mouse motor nerve endings. Indeed, H7 did not block but instead unmasked the effects of the A_1 adenosine receptor agonist. Moreover, the more specific and potent PKA inhibitor KT5720 does not reproduce the effects of H7, yet appears to be effective at blocking PKA in this system. The present result also agrees with earlier studies which suggested that PKA does not mediate the inhibitory effect of A_1 receptor activation at motor nerve endings in the frog (Hirsh *et al*., 1990) or rat (Sebastião & Ribeiro, 1990). It should be noted that H7 is a nonselective protein kinase inhibitor which blocks PKA, protein kinase C (PKC) and protein kinase G (PKG) with similar potencies (K_i values of 3.0, 6.0 μM, and 5.8 μM, respectively (Hidaka *et al*., 1984). Thus, because H7 neither blocks nor mimics the action of CADO, it is therefore likely that none of these other kinases mediate the effects of A_1 receptor stimulation.

In conclusion, our results suggest that whilst adenosine receptors may be modulated by ongoing protein kinase activity, the inhibition of spontaneous ACh release by adenosine receptor activation does not occur as a consequence of inhibition of PKA, or other protein kinases that are blocked by H7. These kinases include PKC and PKG. Plausible mechanisms for the prejunctional inhibitory effect of adenosine analogues on mepp frequency which have been proposed are: a decrease in the ability of Ca^{2+} to promote the exocytosis process (e.g. Silinsky, 1984, 1986; Silinsky & Solsona, 1992) or a decrease in Ca^{2+} entry *via* Ca^{2+} channels (Hamilton & Smith, 1991). However, the latter explanation appears to be less likely in these present experiments because we have restricted this study to spontaneous ACh release which, in the absence of nerve terminal depolarization, is little influenced by extracellular Ca^{2+}. Thus, an action directly upon the secretory apparatus could best explain the effects of adenosine analogues under conditions in which Ca^{2+} entry *via* Ca^{2+} channels makes little or no contribution to the release process.

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