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A Selective Adenosine Antagonist (8-Cyclopentyl-1,3-dipropylxanthine) Eliminates Both Neuromuscular Depression and the Action of Exogenous Adenosine by an Effect on A_1 Receptors

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SUMMARY

The effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A_1 adenosine receptor antagonist, was studied at frog motor nerve endings in the hope of determining whether the inhibitory effects of exogenous or endogenous adenosine on neurotransmitter release are mediated by an A_1 receptor or the postulated prejunctional "A_3 receptor." These putative A_3 receptors have been reported to have a lower affinity for DPCPX (≫1 nM) than A_1 receptors (50–190 pM) and have been linked to changes in Ca^{2+} translocation. The affinity of DPCPX as an antagonist of exogenous adenosine at frog motor nerve endings was calculated by using the Schild equation and found to range from 25 to 200 pM (n = 12). These values are consistent with the presence of A_1 receptors. The effect of endogenous adenosine as a mediator of prejunctional neuromuscular depression produced by repetitive nerve impulses was fully reversed by 100 pM DPCPX. Neither prejunctional neuromuscular depression produced by endogenous or exogenous adenosine nor the reversal of depression by DPCPX was associated with changes in nerve terminal Ca^{2+} currents. The results demonstrate that endogenous or exogenous adenosine mediates neuromuscular depression in the frog, via an A_1 receptor.

Adenosine has long been implicated as an essential mediator of biological functions in both neuronal and non-neuronal cells (1–6). Recently, however, this purine has achieved additional status as a consequence of the molecular cloning of specific adenosine receptors (7–10). The first cloned adenosine receptors, A_1 and A_2, have been suitably named to coincide with the reported physiological effects of adenosine. Most recently, an A_3 receptor has been cloned (10). The action of extracellular adenosine at the cloned A_3 receptor was found to be insensitive to conventional antagonists possessing the xanthine structure and to be associated with decreases in cAMP accumulation. Because of their high level of expression in testes, A_3 receptors have been suggested to have a possible role in reproduction (10).

A major controversy concerning the A_3 adenosine receptor nomenclature originates from studies on a tissue where endogenous adenosine exerts a major physiological function, namely the skeletal neuromucular junction (11–14). Before the molecular cloning of the A_3 receptor, it had been suggested that the inhibitory effects of adenosine at frog motor nerve endings are mediated via a prejunctional "A_3 receptor," a receptor linked to changes in Ca^{2+} entry and/or Ca^{2+} metabolism (15). This postulated prejunctional A_3 receptor is a different protein than the cloned A_3 receptor (10), producing unfortunate confusion regarding the adenosine receptor nomenclature based on molecular biological studies.

The proponents of the prejunctional A_3 receptor have based the preponderance of their evidence on the affinity of the highly selective adenosine receptor antagonist DPCPX (16) at frog motor nerve endings (17, 18). DPCPX has been reported to have a lower affinity as a competitive antagonist of adenosine at frog motor nerve endings (35 nM) (17, 18) than is observed for interactions between adenosine and A_1 receptors (50–190 pM) (16). We recently found that adenosine does not inhibit ACh release as a consequence of Ca^{2+} entry at frog motor nerve endings (19), causing us to question the original model for the coupling of the A_3 receptor to Ca^{2+} channels in the frog (15). We thus decided to evaluate the affinity of DPCPX as an antagonist of both endogenous and exogenous adenosine at frog motor nerve endings, in the hope of determining whether the presence of an A_1 or A_3 receptor provides the most parsimonious

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ABBREVIATIONS: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ACh, acetylcholine; EPP, end-plate potential.

explanation for the behavior of adenosine at motor nerve endings. To this end, we used electrophysiological measurements of physiological ACh release (i.e., EPPs) in conjunction with simultaneous measurements of nerve terminal Ca^{2+} currents. We also tested the suggestion that adenosine derivatives released together with the neurotransmitter ACh act as endogenous mediators of neuromuscular depression via actions on A_1 adenosine receptors (12, 20). A preliminary report of some of this work has been published (21).

Materials and Methods

Overview. Cutaneous pectoris nerve muscle preparations with their attached nerve supply were dissected from frogs (*Rana pipiens*) and superfused with flowing Ringer solution. Suprathreshold electrical stimuli were applied to the proprialis pectoris nerve trunk, via a suction electrode, at frequencies ranging from 0.05 to 10 Hz. Evoked responses (Ca^{2+} currents and EPPs) were recorded using a conventional high-input impedance microelectrode preamplifier purchased from WP Instruments or from Axon Instruments (Axoclamp 2A). Responses were averaged using an IBM AT-compatible microcomputer, TL-1 interface, and pCLAMP software (Axon Instruments, Foster City, CA). Printed copies of the digitalized traces were made on an X-Y plotter or a LaserJet printer (Hewlett Packard Series III). Generally, ASCII files from pCLAMP were first imported to a spreadhsheet program for data organization (Quatro Pro; Borland) before being imported to Sigma Plot 4.0 (Jandel) for printing.

To determine the effects of exogenous adenosine, we stimulated the motor nerve at low frequencies (0.01–0.3 Hz, depending upon the composition of solutions). These low frequencies minimized the neuromuscular depression that ensues with repetitive nerve stimulation. The absence of effects of endogenous adenosine during low frequency stimulation was confirmed in control experiments in which neither DPCPX nor adenosine deaminase affected ACh release. (e.g., Fig. 1) The effect of endogenous adenosine as a mediator of neuromuscular depression was studied at higher frequencies of nerve stimulation (0.05–10 Hz, depending on the composition of solutions), under conditions in which the EPP decreased progressively during continuous nerve stimulation.

Measurements of ACh release and Ca^{2+} currents. Intracellular recordings of EPPs and perineural recordings of Ca^{2+} currents through N-type channels were made using methods previously used in this laboratory (19, 22). Briefly, Ca^{2+} currents were recorded from within the perineurium, after blockade of a proportion of the K^+ channels, using microelectrodes (5–15-mΩ resistance) filled with normal Ringer solution. The perineural electrode was positioned under visual control near small axon bundles at the termination of the myelin sheaths and within 50 μm of the intracellular recording electrode used for measuring EPPs. The perineurium was then gently penetrated: a steady 2–6-mV deflection (generally negative) was associated with successful positioning of the electrode for recording of perineural currents. These currents are reflective of voltage changes across the extracellular resistance; hence, they are reported as the magnitude of the extracellular voltage change in the figures. Perineural Ca^{2+} currents, when flowing across the extracellular and perineural resistances, generally produced a 0.8–3 mV voltage change across the resistance between recording and reference electrodes. Current amplitudes were highly sensitive to electrode position, however. Indeed, in some experiments a decline of both the Na^+ and Ca^{2+} currents was observed; such experiments were not included in the data reported herein (for further details of the particular current waveforms and potential sources of contamination of Ca^{2+} currents, see Refs. 19, 23, and 24). Because adenosine does not affect the size of the miniature EPP, effects of adenosine on EPP amplitudes are due to effects of this purine on evoked ACh release.

Composition of solutions. The normal Ringer solution contained 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM HEPES, pH 7.2–7.4, and 4 mg/liter tubocurarine chloride to reduce the EPPs below the threshold for action potential generation. A modified Ringer solution (0.9 mM $CaCl_2$, 10 mM $MgCl_2$) containing K^+ channel blockers (100 μM diaminopyridine and 250 μM tetraethylammonium) was used for the experiments in which Ca^{2+} currents were measured simultaneously with EPPs (Ca^{2+} current Ringer solution). These concentrations of diaminopyridine and tetraethylammonium were chosen to minimize depletion of ACh quanta (23) yet allow for the detection of both increases and decreases in Ca^{2+} currents as the extracellular Ca^{2+} concentrations were changed accordingly (19).[1] In Ca^{2+} current Ringer solution, the number of ACh quanta released by a nerve impulse ranged from 344 to 569, with a mean of 457 ± 23.8 (mean ± standard error, n = 22), as calculated by the tubocurarine method (22). This higher level of ACh release allowed the phenomenon of prejunctional neuromuscular depression to be observed at very low stimulation rates (e.g., 0.05 Hz; see Fig. 3).

Drugs were obtained from Research Biochemicals Inc. (DPCPX) or the Sigma Chemical Co. (all other drugs).

Delivery of solutions. Normal Ringer solutions were applied continuously by superfusion using a flow pump for both delivering fresh Ringer solution and removing the effluent. Adenosine was dissolved in Ringer solution and applied by this global bath superfusion. For most experiments with DPCPX, this antagonist was dissolved in Ringer solution of the same composition as the bath superfusion solutions and was applied by rapid local superfusion ("fast-flow" delivery) from a series of 300-μm-diameter glass flow tubes (25). The tubes were gravity fed from a syringe reservoir. The latency from the opening of the tap to the beginning of a discernible postjunctional depolarization was 50–100 msec when tested with 100 μM ACh in the flow tube.

Statistical methods and the determination of the equilibrium dissociation constant for DPCPX. Statistical procedures were as described previously (19, 22). In many instances appropriate numbers of evoked responses were averaged to reduce the coefficient of variation (i.e., the standard deviation/mean) to <5%. At the highest level of ACh release in Ca^{2+} current Ringer solution, secretion was high enough so that small differences between individual EPPs were statistically significant.

The K_d for DPCPX was calculated by the Schild equation (26), (dose ratio − 1) = [DPCPX]/K_d. In this equation, dose ratio refers to the ratio of concentrations of adenosine that produce matching levels of ACh release in the presence and absence of DPCPX. In some experiments, estimates were made from pairs of matching levels of ACh release (i.e., EPPs; see Fig. 1). In other experiments, complete Schild plots using dose ratios as high as 100 were constructed (e.g., Fig. 2).

Results

Affinity of DPCPX as an antagonist of exogenous adenosine and evidence for an A_1 receptor at frog motor nerve endings. Fig. 1 shows the results of a typical experiment in which the affinity of DPCPX as an antagonist of exogenous adenosine was estimated. Fig. 1, *upper*, depicts a plot of the EPP amplitude, which directly reflects the level of evoked ACh release, against time. Representative averaged EPPs at corre-

[1] R. Sanger Redman and E. M. Silinsky, unpublished observations.

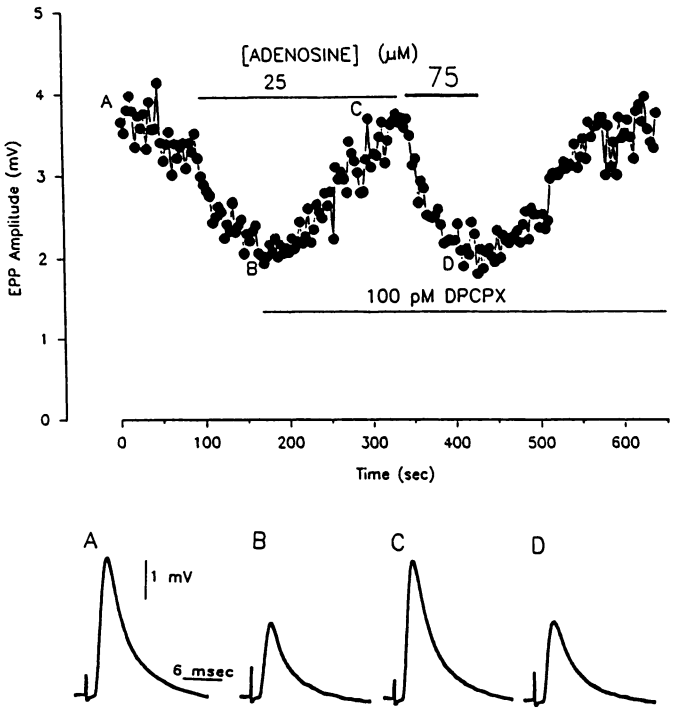

Fig. 1. Determination of the K_d for DPCPX as an antagonist of exogenous adenosine. *Upper*, graph of peak EPP amplitude plotted against time. *Lower*, averaged EPPs beginning at the indicated parts of the plot (A–D) (0.2-Hz nerve stimulation). A, Control EPP = 3.5 mV (average of first 20 EPPs); B, EPP in 25 μM adenosine = 2 mV (n = 5 EPPs); C, EPP in 25 μM adenosine plus 100 pM DPCPX = 3.5 mV (n = 7 EPPs); D, EPP in 75 μM adenosine plus 100 pM DPCPX = 2 mV (n = 5 EPPs). Note the elimination of the inhibitory effect of 25 μM adenosine (B) by 100 pM DPCPX (C) and the restoration of a matching level of inhibition by increasing the adenosine concentration to 75 μM in the presence of 100 pM DPCPX (D).

sponding times during the experiment are also depicted (Fig. 1, *lower*, A–D). These first experiments were performed at low frequencies of nerve stimulation (0.2 Hz), to minimize neuromuscular depression. In the experiment of Fig. 1, after a stable control level of ACh release was obtained (Fig. 1A) superfusion with adenosine (25 μM) inhibited ACh release to 57% of the control level (Fig. 1B). This concentration of adenosine was just submaximal for the presynaptic inhibition of ACh release (maximal inhibitory effects of adenosine were generally 50% of control level and observed with concentrations ranging from 25 to 50 μM adenosine) (13, 21). During the continuous application of 25 μM adenosine, 100 pM DPCPX fully antagonized the inhibitory effect of adenosine in this experiment (Fig. 1C).

The high potency of DPCPX in experiments such as those shown in Fig. 1C suggests that DPCPX has high affinity as an antagonist of adenosine. To determine the K_d for DPCPX, we compared matching levels of inhibition by adenosine in the presence and absence of DPCPX. As Fig. 1D shows, increasing the concentration of adenosine to 75 μM in the presence of 100 pM DPCPX again inhibited ACh release (Fig. 1D) to the level produced by 25 μM adenosine in the absence of DPCPX (Fig. 1B). Inserting these concentrations of adenosine and DPCPX into the Schild equation (see Materials and Methods for justification) yields [(75 μM/25 μM) − 1] = 100 pM/K_d. The K_d for DPCPX was thus 50 pM in this cell.

Fig. 2 shows a complete Schild plot. The plot was linear, with a slope of ≃ 1 (see the legend to Fig. 2 for details). The K_d, as estimated from the DPCPX concentration at which (dose ratio − 1) = 1, was 0.1 nM (100 pM), an estimate that agrees well with the K_d values calculated from individual matching points. Partial Schild plots were constructed for individual cells in two other experiments, and the K_d values were found to be independent of dose ratios. These results demonstrate that DPCPX is a competitive inhibitor of exogenous adenosine at frog motor nerve endings. The K_d in all 12 experiments ranged from 25 pM to 200 pM.

Effects of DPCPX on the action of endogenous adenosine and elimination of neuromuscular depression by A_1 receptor antagonism. We and others have found preliminary evidence suggesting that endogenous adenosine may participate in neuromuscular depression produced by repetitive nerve impulses (21, 27). It would be of interest to investigate the extent of involvement of adenosine in neuromuscular depression and whether these effects of endogenous adenosine are also mediated by A_1 receptors. To test this possibility, we examined the effect of DPCPX during neuromuscular depres-

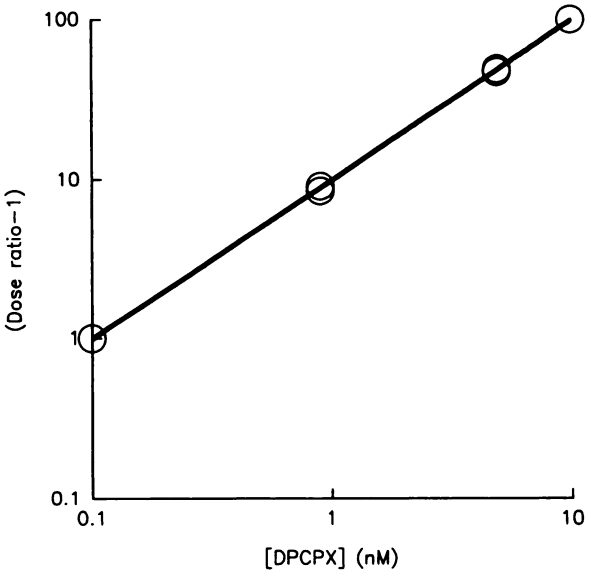

Fig. 2. Schild plot for DPCPX as an antagonist of exogenous adenosine. *Ordinate*, log (dose ratio − 1); *abscissa*, log [DPCPX]. This experiment represents pooled data from four cells from the same animal. The K_d for DPCPX was 100 pM (0.1 nM) in this experiment, as calculated either from the individual data points or from the DPCPX concentration at which (dose ratio − 1) = 1 (see Ref. 26). The estimated slope of the linear regression line was 0.999 ± 0.008 (mean ± 1 SEM). The intercept was 0.994 ± 0.006. The correlation coefficient, the coefficient of determination, and the adjusted coefficient of determination all had values of 1. In two other experiments, partial Schild plots obtained at lower dose ratios with individual cells gave results similar to those shown in this figure, with the estimate of K_d being independent of dose ratio. The K_d values for DPCPX varied from 50 pM to 200 pM in all 12 experiments.

sion. Fig. 3, *Control*, shows the average EPP in response to low frequency stimulation (0.1 Hz). Continuous nerve stimulation at higher frequencies of stimulation (1.0 Hz) produced a prejunctional neuromuscular depression; the EPP was reduced to approximately 50% of control after 1–2 min of stimulation due to presynaptic reduction of ACh release during repetitive nerve stimulation (28, 29). DPCPX (100 pM) applied during neurally evoked prejunctional depression completely eliminated depression, much as 100 pM DPCPX eliminated the inhibitory effects of exogenous adenosine in the absence of neuromuscular depression. In a total of eight experiments, 100 pM DPCPX restored ACh release to 105 ± 3% (mean ± standard error) of the initial level. DPCPX had no significant effect on ACh release at low frequency of nerve stimulation in the absence of neuromuscular depression in this frog preparation (see Fig. 1, *top*). In other experiments, we found similar reversals of neuromuscular depression when preparations were superfused with exogenous adenosine deaminase (21), an enzyme that degrades adenosine to its inactive derivative inosine. Furthermore, the inhibitory effect of exogenous adenosine was occluded during prejunctional depression (21) (data not shown). These results suggest that the action of DPCPX to prevent neuromuscular depression is related to its selective effects on adenosine receptors in preventing endogenously released adenosine from activating the prejunctional adenosine receptor.

To test whether changes in Ca^{2+} entry are responsible for neuromuscular depression produced by endogenous adenosine, we performed the remaining experiments in solutions containing K^+ channel blockers so that Ca^{2+} currents (Fig. 4, *lower*) could be measured simultaneously with EPPs (Fig. 4, *upper*) (see Materials and Methods). Note that, although 100 pM DPCPX completely eliminated neuromuscular depression of ACh release during repetitive nerve stimulation, neither neuromuscular depression nor the elimination of depression by

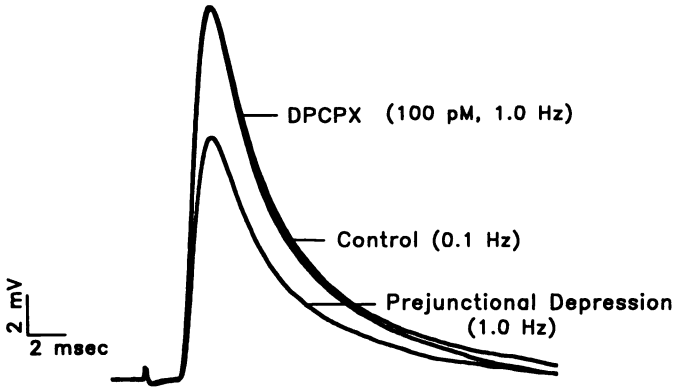

Fig. 3. Elimination of presynaptic neuromuscular depression by the A_1 adenosine receptor antagonist DPCPX (100 pM). The motor nerve trunk was stimulated for approximately 2 min at 0.1 Hz (*Control*) before the stimulation rate was increased to 1.0 Hz. Each of the three traces represents an average EPP (5–10 stimuli) for each of the experimental conditions. *Trace* labeled *Prejunctional Depression*, depression produced by the increase in stimulation frequency. With continued stimulation at 1.0 Hz, application of 100 pM DPCPX completely eliminated prejunctional depression. The application of these drugs in a nondepressed state produced no significant effect (see end of Fig. 1, *upper*).

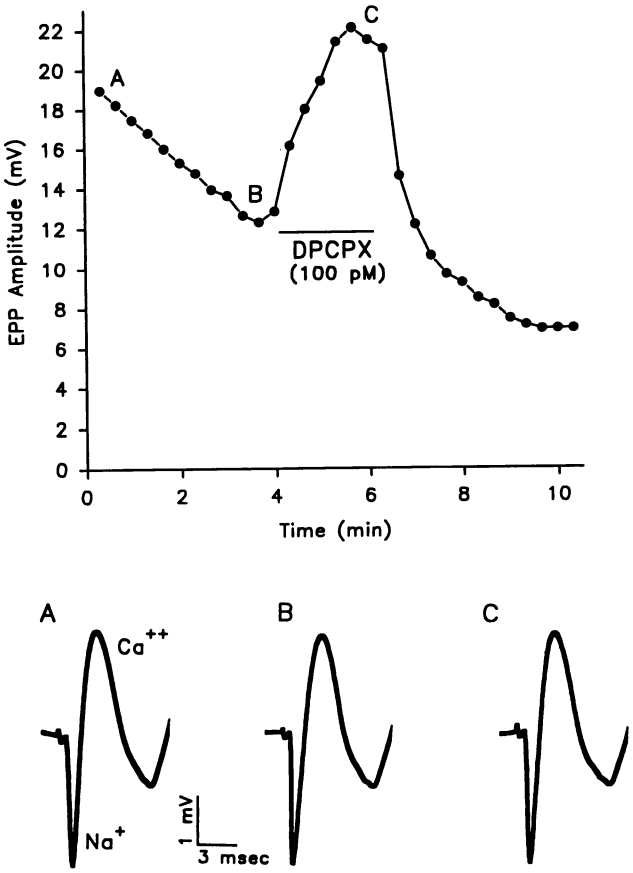

Fig. 4. Simultaneous measurements of ACh release reflected as EPP amplitudes (*upper*) and perineural Ca^{2+} currents (*lower*) during neuromuscular depression and during the actions of DPCPX. Nerve was stimulated at 0.05 Hz for the duration of the experiment (see Materials and Methods). Note the blockade of neuromuscular depression by 100 pM DPCPX without changes in the corresponding Ca^{2+} currents during depression or recovery. The Ca^{2+} current in the nerve ending is the large upward deflection at the end of the traces. The downward spike preceding the Ca^{2+} current represents the Na^+ current originating from the junction of the myelinated and nonmyelinated axon. Note that only the initial components of the current traces are shown, although the currents from several experiments demonstrated repetitive firing (for additional details, see Refs. 19, 23, and 24). *Calibration bar*, voltage change of 1.0 mV produced by current flow across the perineural resistances.

DPCPX was associated with concomitant changes in Ca^{2+} currents under these conditions (Ca^{2+} currents are upward deflections in the electrophysiological traces of Fig. 4, *lower*). The effects of DPCPX on ACh release were reversible; after the cessation of superfusion with DPCPX, depression of EPP amplitudes continued along its original time course. In contrast to the absence of effect of endogenous adenosine on Ca^{2+} currents (Fig. 4), increasing or decreasing the extracellular Ca^{2+} concentration increased or decreased Ca^{2+} currents under the same experimental conditions (see Materials and Methods and Ref. 19).

Discussion

The high affinity of the selective A_1 receptor antagonist DPCPX and the absence of effects of endogenous or exogenous

adenosine on Ca^{2+} currents are not consistent with the presence of A_3 receptors linked to changes in Ca^{2+} entry in frog motor nerve endings (16–18). These results support the suggestion that adenosine, via an action on the presynaptic A_1 adenosine receptor, inhibits ACh release not by blocking Ca^{2+} channels but by impairing the ability of Ca^{2+} to promote the secretory process in the frog (19, 22, 30). Phospholipase C has recently been suggested to mediate the effects of adenosine (31), but the evidence for the involvement of this lipase is scant and other interpretations of the data are possible.

The reason for the previously reported low affinity of DPCPX for frog motor nerve (17) is unknown. It is possible that the use of twitch tension measurements in frog sciatic nerve/sartorius muscle preparations (17) did not provide an accurate measure of the presynaptic interactions between adenosine and DPCPX, due to the high safety factor of neuromuscular transmission observed when muscle contraction is used to monitor presynaptic phenomena. In addition, substantial tonic levels of extracellular adenosine were present in the absence of nerve stimulation in the previous studies using frog neuromuscular junctions (14, 27); this too could affect quantitative measurements of the affinity of DPCPX for the presynaptic adenosine receptor. With respect to other species, previous results in mammalian muscle are also consistent with the presence of a prejunctional A_1 receptor (32, 33), although the transduction mechanism may differ between frog and rat motor nerve (32).

The effects of endogenous adenosine merit further discussion. In the only full study addressing the issue of adenosine and neuromuscular depression in frog, the effects of endogenous adenosine appeared to be very modest (27), possibly because basal adenosine levels were already high (27). In our present study we have minimized basal effects of adenosine and used a highly selective A_1 receptor antagonist (16); the results suggest that neuromuscular depression is caused by the release of endogenous adenosine. Our results are consistent with a model in which ATP is released from motor nerve endings in stoichiometric amounts with ACh (12) and, after degradation to adenosine (13, 14), is the exclusive mediator of neuromuscular depression at normal levels of ACh output (21). In support of this, we found that α,β-methylene-ADP (50 μM), an inhibitor of ATP degradation in this species (14), completely prevented neuromuscular depression (34), suggesting that adenosine derived from presynaptic sources is released as the parent nucleotide ATP and must then be hydrolyzed to adenosine to produce its presynaptic inhibitory effect (see also Refs. 13 and 14).

Regardless of the nature of the coupling mechanism or the importance of endogenous adenosine, there does not appear to be sufficient evidence to attribute the prejunctional effects of adenosine at frog motor nerve endings to an action at a hypothetical A_3 receptor. It is suggested that the A_3 receptor nomenclature be reserved for proteins similar to the A_3 receptor recently found by molecular cloning techniques (10, 35).

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