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Adenosine inhibition via A_1 receptor of N-type Ca^{2+} current and peptide release from isolated neurohypophysial terminals of the rat

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Effects of adenosine on voltage-gated Ca^{2+} channel currents and on arginine vasopressin (AVP) and oxytocin (OT) release from isolated neurohypophysial (NH) terminals of the rat were investigated using perforated-patch clamp recordings and hormone-specific radioimmunoassays. Adenosine, but not adenosine 5′-triphosphate (ATP), dose-dependently and reversibly inhibited the transient component of the whole-terminal Ba^{2+} currents, with an IC_{50} of 0.875 μM. Adenosine strongly inhibited, in a dose-dependent manner (IC_{50} = 2.67 μM), depolarization-triggered AVP and OT release from isolated NH terminals. Adenosine and the N-type Ca^{2+} channel blocker ω-conotoxin GVIA, but not other Ca^{2+} channel-type antagonists, inhibited the same transient component of the Ba^{2+} current. Other components such as the L-, Q- and R-type channels, however, were insensitive to adenosine. Similarly, only adenosine and ω-conotoxin GVIA were able to inhibit the same component of AVP release. A_1 receptor agonists, but not other purinoceptor-type agonists, inhibited the same transient component of the Ba^{2+} current as adenosine. Furthermore, the A_1 receptor antagonist 8-cyclopentyltheophylline (CPT), but not the A_2 receptor antagonist 3, 7-dimethyl-1-propargylxanthine (DMPGX), reversed inhibition of this current component by adenosine. The inhibition of AVP and OT release also appeared to be via the A_1 receptor, since it was reversed by CPT. We therefore conclude that adenosine, acting via A_1 receptors, specifically blocks the terminal N-type Ca^{2+} channel thus leading to inhibition of the release of both AVP and OT.

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ATP is known to affect several biological functions (see reviews by Burnstock, 1978; El-Monatassim *et al.* 1992) including neurotransmission (Ribeiro, 1978; Burnstock, 1978, 1996; White, 1988; Fieber & Adams, 1991; Evans *et al.* 1992; Edwards & Gibb, 1993; Wu & Saggau, 1994; Zimmermann, 1994; Kennedy *et al.* 1996), secretion (Diverse-Pierluissi *et al.* 1991; Gandia *et al.* 1993; Currie & Fox, 1996; Harkins & Fox, 2000) and vascular contraction (Kolb & Wakelam, 1983; Katsuragi & Su, 1980). The modulation by ATP of pre-synaptic ion channels (Zimmermann, 1994) via specific purinergic receptors, for example, could result in changes in subsequent release of neurotransmitters from the pre-synaptic site and, as a consequence, on the functional effect at the post-synaptic site.

ATP is rapidly hydrolysed by ecto-nucleotidases (Gordon *et al.* 1989) even at pre-synaptic sites (Thirion *et al.* 1996) to adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine. Adenosine, the final hydrolysed metabolite of ATP (Gordon *et al.* 1989; Kennedy *et al.* 1996; see review by Rathbone *et al.* 1999), is another strong neuromodulator that may interact with another group of specific receptors at pre- (White, 1988; Scanziani *et al.* 1992; Yawo & Chuhma, 1993; Umemiya & Berger, 1994; Wu & Saggau, 1994) or post-synaptic sites (Rathbone *et al.* 1999). These adenosine receptors belong to a family of receptors including A_1, A_2 and A_3 subtypes (Reppert *et al.* 1991) that are distinct from those purinergic receptors specifically activated by ATP (see reviews by Dalziel & Westfall, 1994; Fredholm *et al.* 1994; Rathbone *et al.* 1999). Despite an accumulation of evidence that adenosine acts as an auto-modulator via these pre-synaptic receptors to exert negative feedback, no direct evidence has demonstrated mechanisms by which it modulates such pre-synaptic functions. In order to address this issue, we utilized the rat NH as an example of central 'pre-synaptic' nerve terminals, since endogenous ATP was reported to be co-stored with the neuropeptides AVP and OT in their large neurosecretory vesicles (Troadec et al., 1998; Sperlagh *et al.* 1999) and hydrolysed by ecto-nucleotidases to adenosine (Thirion *et al.* 1996).

Ca^{2+} entry into pre-synaptic nerve terminals is required for triggering exocytotic release of neurotransmitters/

hormones (Llinas *et al.* 1981; Augustine *et al.* 1985; Turner *et al.* 1993; Reuter, 1995). It has been established that during depolarization–secretion coupling in NH terminals, Ca^{2+}, acting as a second messenger, flows into these nerve terminals via voltage-gated Ca^{2+} channels and consequently evokes neurohormone release (Brethes *et al.* 1987; Dayanithi *et al.* 1987; Wang *et al.* 1993). There are four subtypes of high voltage-threshold Ca^{2+} channels in the rat NH terminals: L- (Lemos & Nowycky, 1989), N- (Wang *et al.* 1992*b*), P/Q- (Wang *et al.* 1997) and R-types (Wang *et al.* 1999). While the L- and N-type Ca^{2+} channel currents play equivalent roles in AVP and OT release, Q-and R-type Ca^{2+} channel currents preferentially control AVP *vs.* OT release (Wang *et al.* 1997, 1999).

We measured voltage-gated ion channel currents and hormone release in order to determine if they were affected by exogenously applied adenosine or specific agonists/antagonists of purinoceptors (Thirion *et al.* 1996; Troadec *et al.* 1998; Sperlagh *et al.* 1999; Loesch *et al.* 1999; Lemos & Wang, 2000). We found that adenosine exerted significant effects on both N-type Ca^{2+} channel currents (Wang & Lemos, 1995) and peptide release from these terminals.

METHODS

Electrophysiology

Isolation of nerve terminals. Experiments were conducted on freshly dissociated NH nerve terminals of male adult (6–8 weeks) CD rats (Charles River Laboratory, Boston, MA, USA). Briefly, as previously described (Cazalis *et al.* 1987), the rat, after anaesthetization by a high concentration of CO_2, was decapitated (as approved by University of Massachusetts Medical School protocol A-1031), the brain was removed and the pituitary gland then excised. The NH, after being carefully separated from the anterior pituitary and the pars intermedia, was homogenized in the following solution (mM): sucrose, 270; Tris-Hepes, 10; EGTA, 0.01; pH 7.2. The isolated terminals were then placed in a 35 mm Petri dish coated with 0.1 % poly-L-lysine and perfused with normal-Ca^{2+} Locke solution containing (mM): NaCl, 145; KCl, 5; $CaCl_2$, 2.2; $MgCl_2$, 1; Tris-Hepes, 10; glucose, 15; pH 7.3. The nerve terminals could be identified using an inverted microscope equipped with phase and Hoffman-modulated contrast optics (Nikon, Tokyo, Japan). Only terminals 7–9 μm in diameter were chosen for patch-clamping. The electrophysiological experiments were all done at room temperature, 22–24 °C and the release experiments were performed at 37 °C.

Whole-terminal patch-clamp recording. After approximately 1 h of perfusion with normal-Ca^{2+} Locke solution, nerve terminals were then perfused with Ba^{2+} solution (mM): TEA-Cl, 100; NaCl, 30; $BaCl_2$, 5; KCl, 5; $MgCl_2$, 1; Tris-Hepes, 10; glucose, 15; pH 7.3. In these experiments, the pipette solution for amphotericin B-perforated (Rae *et al.* 1991; Lemos & Wang, 1993; Wang *et al.* 1997, 1999), whole-terminal patch-clamp contained (mM): caesium glutamate, 135; TEA-Cl, 20; Tris-Hepes, 10; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 5; pH 7.2, including amphotericin B, 30 μg ml^{-1}. All chemicals were obtained from Sigma (St Louis, MO, USA). Most of the terminals were raised from the bottom of the Petri dish after GΩ seal formation so that the entire area of their membrane would have direct contact with the perfusion medium. While the perforation was developing, the capacitance of the terminal gradually increased. Those terminals with access resistances less than 35 MΩ of the perforated-patch membrane were then chosen for the Ca^{2+} channel current recordings. Using the perforated-patch (Rae *et al.* 1991), 'whole-terminal' configuration (Hamill *et al.* 1981), the amplitude of the Ba^{2+} current (I_{Ba}) was usually stable for more than 30 min without any rundown problems. This allowed us to sequentially apply specific Ca^{2+} channel blockers and test specific agonists/antagonists. The L-type channel is long-lasting and very sensitive to dihydropyridines (DHP) (Nowycky *et al.* 1985; Fox *et al.* 1987; Tsien *et al.* 1988; Lemos & Nowycky, 1989; Wang *et al.* 1992; Wang *et al.* 1997, 1999). The N-, P/Q- and R-type Ca^{2+} channel currents, however, are transient and can be identified pharmacologically by their sensitivity to selective peptide toxins (Nowycky *et al.* 1985; Fox *et al.* 1987; Tsien *et al.* 1988; Lemos & Nowycky, 1989; Turner *et al.* 1992; Mintz *et al.* 1992*a*, *b*; Wang *et al.* 1992*b*; Sather *et al.* 1993; Stea *et al.* 1994; Wheeler *et al.* 1994; Wang & Lemos, 1994; Wang *et al.*1997, 1999; Newcomb *et al.* 1998).

Membrane currents were recorded by an EPC-7 amplifier (List Electronics, Germany) and were filtered at 3 kHz corner frequency, −3 dB, with an 8-pole Bessel filter (902LPF, Frequency Devices Inc., Haverhill, MA, USA) and stored on high-density disks for later analysis. pCLAMP computer software (Axon Instruments, Inc., Union City, CA, USA) and an A–D converter (Scientific Solutions, Solon, OH, USA) were used to generate command voltage potentials. The resistances of soft glass pipettes (Drummond Scientific Co., PA, USA), which had been double-pulled (Model 700C, David Kopf Instruments, Tujunga, CA, USA), Sylgard-coated, and fire-polished (Narishige Scientific Instrument Lab., Tokyo, Japan), were 2–3 MΩ.

Data analysis. pCLAMP computer software (versions 5 and 6) was used for the acquisition and analysis of the data. In most cases, the leakage currents were subtracted from the total currents in order to analyse the pure inward I_{Ba}s. The amplitude of I_{Ba} at the end of the 200 ms steps was considered to be that of the long-lasting component of the I_{Ba} and the difference in amplitude between this component of I_{Ba} and the peak I_{Ba} is considered to be that of the transient I_{Ba} (Lemos & Nowycky, 1989). Data are given as means ± S.E.M. Student's *t* test was used to examine statistical significance of paired or unpaired data, as indicated.

Drugs

Adenosine, ATP, 2-methylthio-ATP tetrasodium (2Met-ATP), 2-chloroadenosine (2-CA), N-0840, DMPGX, CPT and the DHP nicardipine were all purchased from Research Biochemicals International (Natick, MA, USA). The polypeptide toxins SNX-482, ω-conopeptides GVIA and MVIIC utilized in this study were the synthetic versions, prepared by Neurex Pharmaceutical Corporation (Palo Alto, CA, USA; see Wang et al. 1997, 1999). The synthetic version of ω-AgaIVA (Mintz *et al.* 1992*a, b*) was purchased from Peptides International (Louisville, KY, USA).

Assay of peptide release

The nerve endings were prepared as previously described (Cazalis *et al.* 1987; Wang *et al.* 1997). Briefly, rat neurohypophyses were homogenized in a solution containing (mM): sucrose, 270; Tris-Hepes, 10 (pH 7.25); EGTA, 2. The homogenate was centrifuged at 100 *g* for 2 min and the resulting pellet was centrifuged at 2400 *g* for 6 min. The final pellet contains highly purified nerve

terminals. The isolated nerve terminals were loaded onto filters (0.45 μm Acrodisc, Gelman Scientific, Ann Arbor, MI, USA) and perfused at 37°C with Locke solution (Dayanithi *et al.* 1987). Fractions of perfusate were collected at 2 min intervals and the evoked release was triggered by an eight minute long pulse of a depolarizing concentration (50 mM) of KCl. The results are given as AVP or OT release per fraction measured using specific radioimmunoassays (Cazalis *et al.* 1987; Dayanithi *et al.* 1987). The medium before and after the depolarizing period contained (mM): NaCl, 40; $KHCO_3$ 5; *N*-methyl-D-glucamine (NMG)-Cl_2, 100; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; Tris-Hepes, 10 (pH 7.2) with 0.02 % BSA. Depolarization medium contained 50 mM K^+, in which the NMG was reduced to maintain the osmolarity (300–305 mOsmol).

RESULTS

Adenosine dose-dependently inhibits the transient Ba^{2+} currents in NH terminals

We examined the effects of adenosine on voltage-gated Ca^{2+} channel currents in isolated NH nerve terminals of the rat. There are four subtypes of high threshold-voltage-activated Ca^{2+} channel currents: L, N and P/Q or R in these nerve terminals (Lemos & Nowycky, 1989; Wang *et al.* 1992*b*, 1997, 1999; Wang & Lemos, 1992, 1994). While the L-type current is long lasting, the N-, P/Q- and R- types are transient currents (Lemos & Nowycky, 1989; Wang *et al.* 1992*b*, 1997, 1999). We observed that ATP exerted little influence (−9.2 ± 5.2 %; $n = 3$) on Ba^{2+} current at doses as high as 0.5 mM (Fig. 1*A*) to 1 mM (Wang & Lemos, 1995). In contrast, 0.2–0.7 mM adenosine specifically inhibited (by 61.2 ± 12 %; $n = 3$) the transient part of whole-terminal Ba^{2+} currents (Fig. 1*B* and *C*). Even very low (0.6 μM) concentrations of adenosine displayed a significant inhibitory effect (−27.5 ± 5.3 %; $n = 4$) on the transient component of whole-terminal Ba^{2+} currents (Fig. 2*A*). When increased to 2 μM and above, the effects of adenosine on this transient Ba^{2+} current appear to be saturated at approximately 37 % (36.8 ± 2.9 %; $n = 9$) of controls, with an IC_{50} of 0.875 μM (Fig. 2*B*). This inhibition is reversible upon washing with the adenosine-free control buffer (Figs. 1*C* and 2*A*).

Adenosine dose-dependently inhibits peptide release from NH terminals

In order to determine if the metabolite of ATP, adenosine, could modulate neurosecretion, we measured AVP and OT release, using specific radioimmunoassays, from isolated, perfused NH terminals (Dayanithi *et al.* 1987). Figure 3*A* shows repeated stimulations (S) with 50 mM K^+ that gave, each time, essentially identical AVP release profiles, (basal = 45 ± 6 pg; evoked S1 = 466 ± 12 pg; S2 = 443 ± 15 pg; S3 = 431 ± 9 pg; $n = 5$). The Ca^{2+}-dependent AVP release was strongly inhibited by 200 μM adenosine (Fig. 3*B*; basal = 40 ± 3 pg; evoked S1 = 418 ± 7 pg; S2 = 196 ± 6pg; S3 = 390 ± 15 pg; $n = 5$), but not even higher (500 μM) concentrations were able to completely block the stimulated release (basal = 44 ± 5 pg; evoked S1 = 418 ± 13 pg; S2 = 189 ± 12pg; S3 = 390 ± 12 pg; $n = 5$) (Fig. 3*C*). Furthermore, the maximal proportion (42 %) of release affected by adenosine correlates well with the proportion (35 %) regulated by the N-type Ca^{2+} current (Wang *et al.* 1997, 1999).

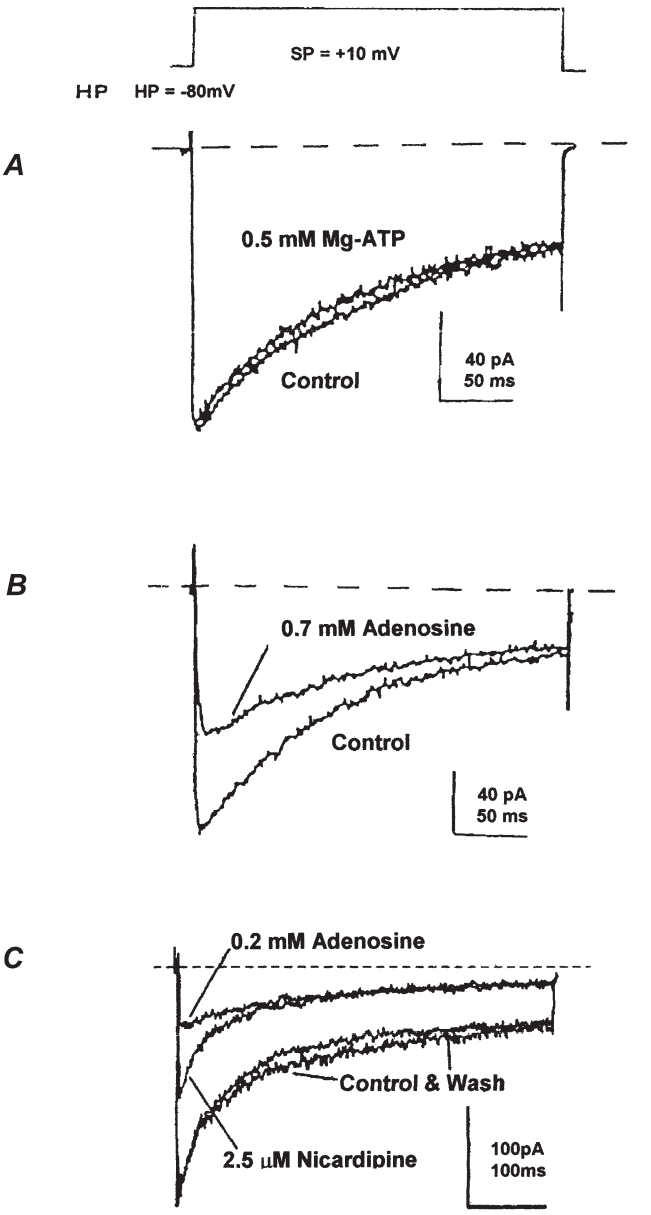

Figure 1. Adenosine inhibits the transient Ba^{2+} current in NH terminals.

The transient component of whole-terminal Ba^{2+} currents elicited from – 80 mV to +10 mV (see protocol above) using the perforated-patch method was strongly and specifically inhibited by 0.7 mM adenosine (*B*), but not by 0.5 mM ATP (*A*). *C*, representative traces of the whole-terminal Ba^{2+} currents also elicited from – 80 mV to +10 mV in a different isolated NH terminal. The L-type Ca^{2+} channel current was first blocked by a saturating concentration (2.5 μM) of nicardipine (Wang *et al.* 1997, 1999). Further application of 0.2 mM adenosine led to a reversible (wash) inhibition of the nicardipine-resistant, transient Ba^{2+} current. Dotted lines represent baseline currents.

Adenosine inhibits the N-type component of Ca^{2+} currents

In an effort to determine which Ca^{2+} current subtype is specifically blocked by adenosine, pharmacological protocols known to differentiate between the terminal Ca^{2+} channel types (Lemos & Nowycky, 1989; Wang *et al.* 1992*b*, 1997, 1999) were utilized. As already shown in Fig. 1*C*, the DHP Ca^{2+}-channel antagonist nicardipine (2.5 μM) selectively inhibited the long-lasting component of the Ba^{2+}-current in these isolated NH terminals, in agreement with previous reports (Lemos & Nowycky, 1989; Wang *et al.* 1997, 1999). In the presence of 2.5 μM nicardipine, the total peak NH Ba^{2+} current was decreased from 100 ± 34.3 % (n = 7) to 71.1 ± 9.1 % (n = 4).

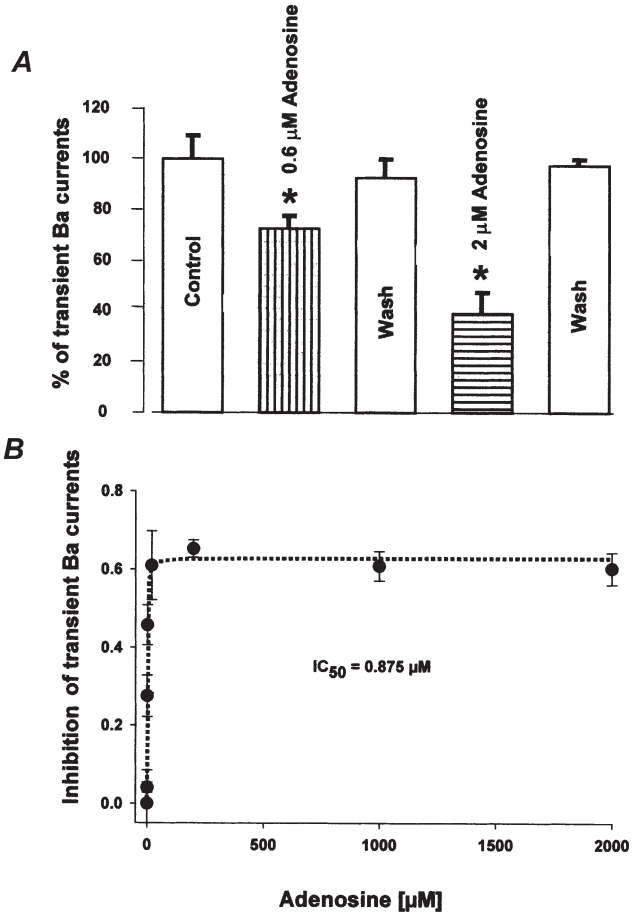

Figure 2. Adenosine dose-dependently inhibits the transient Ba^{2+} current in nerve terminals

A, the isolated transient component (see Fig. 1*C*) of NH Ba^{2+} currents was dose-dependently and reversibly inhibited by low concentrations (0.6 – 2 μM) of adenosine. Furthermore, the inhibitory effects of adenosine on the isolated transient current were reversible upon washing (Wash). * Significant ($P < 0.05$) differences from controls in this and all subsequent figures. *B*, dose–response curve for the effects of adenosine on the transient Ba^{2+} current in the nerve terminals indicates an IC_{50} of 0.875 μM. Dotted line represents Lineweaver-Burk fit (r^2 =0.97) to the data (expressed as means ± S.E.M., n = 3–5).

Subsequent addition of 2 μM adenosine still led to rapid inhibition of a large proportion (36.5 ± 10.3 %; n = 7, $P < 0.05$) of the remaining transient component of the Ba^{2+}-current, suggesting that the L-type Ca^{2+} current was not involved in the inhibition by adenosine.

Furthermore, as shown in Fig. 4*A*, after blocking the L-type current (control), upon addition of the specific N-type channel blocker ω-conotoxin GVIA a large portion (69.5 ± 6.5 %; n = 5) of the NH transient Ba^{2+} current was reduced. Subsequent addition of even saturating (0.5–2 mM) concentrations of adenosine (n = 6) did not significantly affect the remaining current. Addition of 450 nM ω-AgaIVA could, however, completely block (by 98.1 ± 2.1 %) the remaining P/Q type component of the Ba^{2+} current (Wang *et al.* 1997, 1999).

In other experiments in the presence of nicardipine, addition of saturating (0.5–2 mM) concentrations of adenosine could induce a further decrease in the peak NH Ba^{2+} current from 71.1 ± 9.1 % (n = 4) to 34.6 ± 20.3 % (n = 7, $P < 0.05$). Furthermore, application of even a saturating concentration (800 nM) of ω-conotoxin GVIA (Wang *et al.* 1992*b*) failed to significantly (n = 4, $P > 0.05$) inhibit the nicardipine- and adenosine-resistant component of the NH Ba^{2+} current, which was changed from 34.6 ± 20.3 % in the presence of nicardipine and adenosine (n = 7) to 25.8 ± 10.2 % in the presence of ω-conotoxin GVIA. Application of P/Q-type channel (Mintz *et al.* 1992 *a*, *b*) blockers ω-AgaIVA (450 nM) or ω-MVIIC (100 nM), however, could eventually reduce the remaining Ba^{2+} current from 25.8 ± 10.2 % (n = 4) to 2.2 ± 2.0 % (n = 3).

This inhibition of the nicardipine- and adenosine-resistant transient Ca^{2+} current component by both low concentrations of SNX-482 (by 95 %; n = 2), a specific R-type Ca^{2+} channel blocker (Wang *et al.* 1999), and high concentrations of ω-AgaIVA (Fig. 4*B*; n = 4) lead us to conclude that adenosine cannot inhibit the Q- or R-type channel (Wang *et al.* 1997, 1999). In addition, neither ATP nor adenosine had any significant effect on the L-type current (see above). Finally, since the effects of ω-conotoxin GVIA and adenosine are not additive, it appears that adenosine specifically blocks only the N-type channel in NH terminals (Wang & Lemos, 1995).

Adenosine only inhibits the N-type component of AVP release

In order to check for the involvement of N-type Ca^{2+} channels in the inhibition of AVP release by adenosine, we performed two different sets of experiments. Figure *A* and *B* shows that the high K^+-evoked release (395 ± 9 pg; n = 4) was significantly ($P < 0.01$) inhibited (198 ± 20 pg) by the N-type channel antagonist, ω-conotoxin GVIA (Kasai *et al.* 1987; Hirning *et al.* 1988; Wang *et al.* 1997) and that 200 μM adenosine did not further inhibit AVP release (211 ± 12 pg). However, additional inhibition could

be achieved (49 ± 8 pg) by application of L- and P/Q-type channel blockers (nicardipine and ω-AgaIVA). Similarly, we have confirmed these results by reversing the order of application of adenosine, and the L- and N-type Ca^{2+} channel blockers (see Fig. 5*C* and *D*). The high K^+-evoked AVP release (341 ± 18 pg; $n = 4$) inhibited by adenosine (161 ± 10 pg) and further inhibited (90 ± 5 pg) by nicardipine, an L-type channel blocker, was not affected (73 ± 6 pg) by GVIA, an N-type channel blocker.

A_1 receptor mediates the inhibition by adenosine of the N-type Ca^{2+} current

In order to determine which receptor subtype is involved in inhibition of the whole terminal Ca^{2+} channels, we then examined the effects on the Ba^{2+} currents of several purinergic receptor subtype agonists, such as the adenosine A_1 receptor agonist 2-chloroadenosine (2-CA; Fredholm *et al.* 1994), the A_2 receptor agonist 2-phenylamino-adenosine (White, 1988), and the P2Y receptor agonist 2-methylthio-ATP tetrasodium (2Met-ATP) (Zimmerman, 1994).

2Met-ATP (Fig. 6; $n = 4$), 2-phenylamino-adenosine (data not shown; $n = 2$), and Mg-ATP (Figs 1*A* and 6*B*; $n = 3$) were all ineffective on the whole-terminal Ba^{2+} currents resistant to nicardipine. In contrast, the A_1 agonist 2-CA, at 20–100 μM concentrations, had effects (−64.7 ± 5 %; $n = 5$) similar to those of adenosine (−61.7 ± 12.2 %; $n = 3$) on the transient Ba^{2+} current in the nerve terminals (Fig. 6*A* and *B*). In addition, as shown in Fig. 7, only the A_1 receptor antagonist CPT ($n = 8$), but not the A_2 receptor antagonist DMPGX ($n = 3$), could reverse the inhibition by adenosine ($n = 9$) of the transient Ba^{2+} current in the nerve terminals. By themselves, neither CPT (−4.1 ± 1.7 %) nor DMPGX (−6.2 ± 8.2 %) had any substantial effect on the transient Ba^{2+} current in the nerve terminals. The receptor agonist/antagonist data thus indicate that the

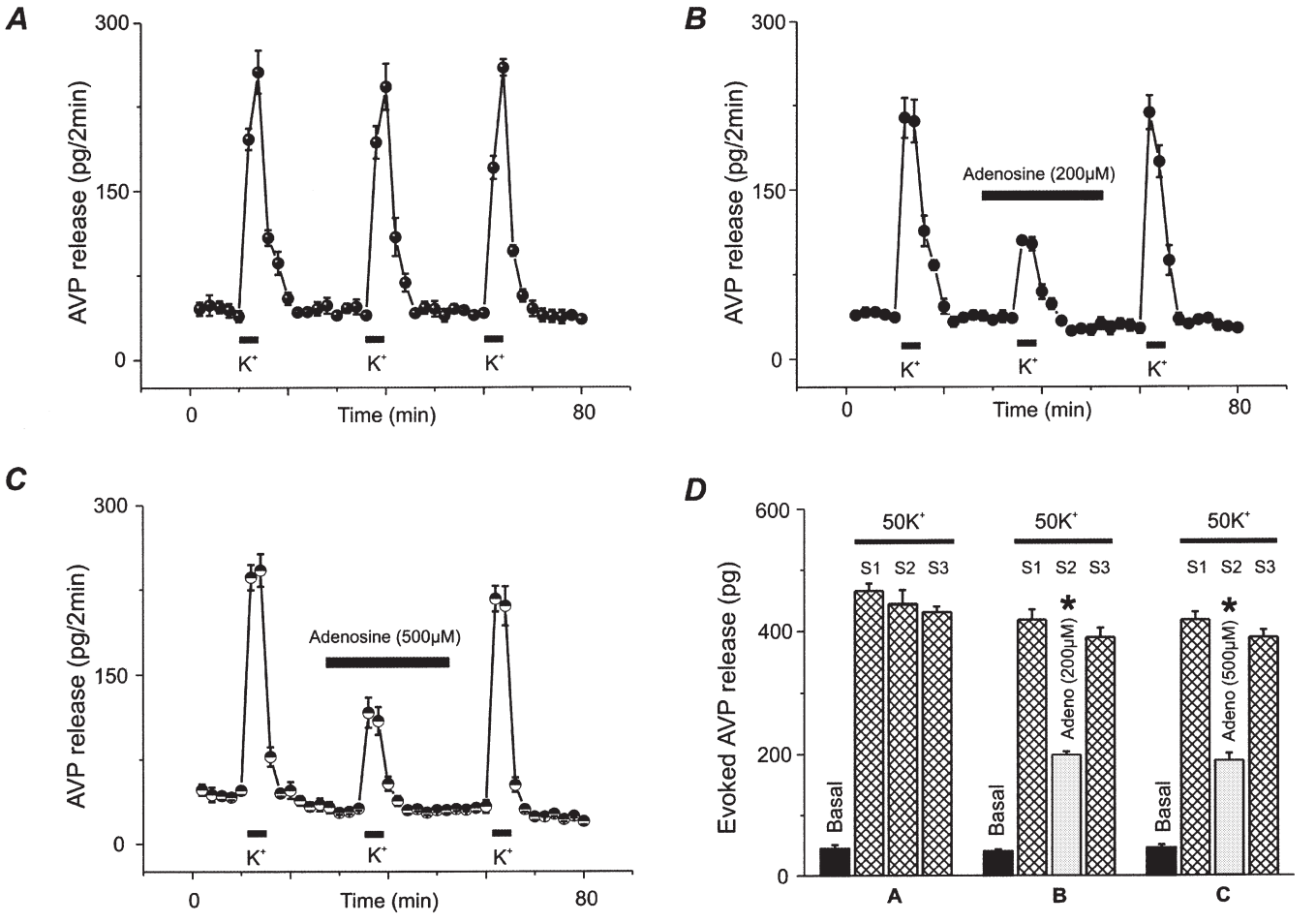

Figure 3. Effects of adenosine on the release of AVP from isolated rat NH nerve terminals

A, AVP release was stimulated (S) for 8 min by 50 mM KCl (K^+) repeated at three regular time intervals. *B*, nerve terminals were challenged with high K^+ either in the absence (S1 & S3) or in the presence (S2) of 200 μM adenosine as indicated by the bar above the data points. All drugs were present for 20 min before, during and after high K^+ challenges. *C*, similarly, in order to further characterize this action on Ca^{2+}-dependent peptide release, 500 μM adenosine was added 20 min before and during the second stimulus (S2). All traces represent the mean of 4–6 experiments ± S.E.M. *D*, bar graph summarizes basal and evoked release for panels *A*, *B* and *C* expressed as means ± S.E.M., $n = 4$–6.

activation of an A_1 receptor mediates the inhibition of the N-type Ca^{2+} channel by adenosine.

A_1 receptor mediates adenosine inhibition of peptide hormone release

In an effort to determine what purinoceptor might mediate the effects of adenosine on AVP and OT release we attempted to block the inhibition by adenosine with various antagonists. Even lower concentrations (20 μM) of adenosine were able to significantly ($P < 0.01$) inhibit both high K^+ stimulated AVP (Fig. 8*A*; basal release = 44 ± 4 pg; 50 mM K^+ = 512 ± 28 pg; plus adenosine = 194 ± 16 pg; $n = 4$;) and OT release (Fig. 8*C*; basal = 80 ± 10 pg; 50 mM K^+ = 712 ± 65 pg; plus adenosine = 204 ± 24 pg; $n = 4$;), and this inhibition could be reversed only by A_1-antagonists, such as CPT (Fig. 8*B* and *D*; recoveries of 93.2 ± 4.8 % for AVP and 87.5 ± 3 % for OT) or N-0840 (84.8 ± 10.7 % recovery; $n = 3$), but not by the A_2-antagonist, DMPGX (2.7 ± 5 %; $n = 3$). Taken together, the release results demonstrate an apparent IC_{50} of 2.67 μM for adenosine. This correlates reasonably well with the electrophysiological data (see above), suggesting that the N-type Ca^{2+} channel component mediates the action of adenosine via A_1-receptors on depolarization-secretion coupling in these central nervous system terminals.

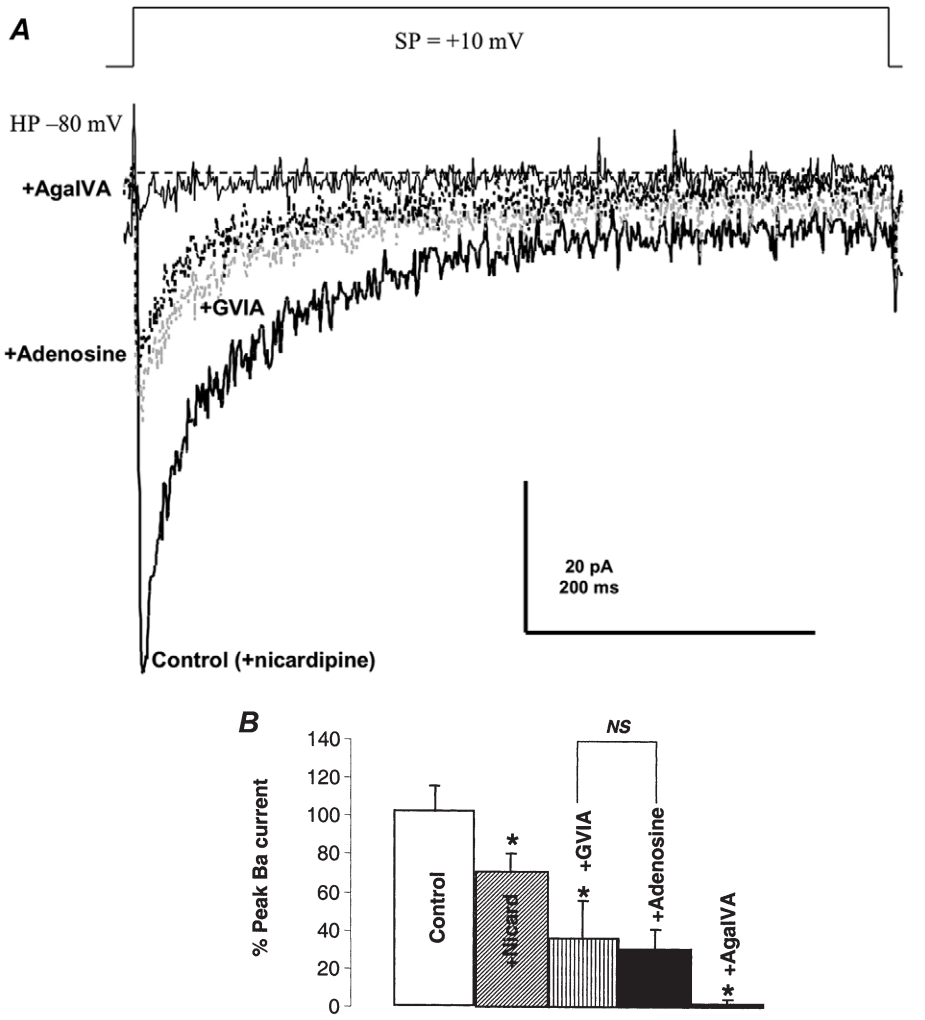

Figure 4. The transient Ba^{2+} current blocked specifically by adenosine is the N-type Ca^{2+} channel

A, the isolated transient Ba^{2+} current was elicited by stepping from −80 to +10 mV (SP) in the presence of 2.5 μM nicardipine (control). Saturating concentrations (800 nM) of the N-type channel blocker ω-conotoxin GVIA (grey, dotted line) were used to block the N-type Ca^{2+} current in this nerve terminal. Not even very high concentrations (2000 μM) of adenosine (black, dotted line) had any significant effect on the ω-conotoxin GVIA-resistant, transient Ba^{2+} current. This nicardipine-, ω-conotoxin GVIA- and adenosine-resistant Ba^{2+} current is, however, sensitive to high concentrations (450 nM) of ω-AgaIVA. *B*, bar graph summarizes multiple ($n = 4$–7) effects observed as in *A*. *NS*, no significant differences between indicated conditions in this and all subsequent figures ($P > 0.05$).

DISCUSSION

This paper reports a role for purines as modulators of both ionic channels and peptide hormone secretion from the isolated nerve terminals of the rat NH. The ATP metabolite, adenosine, appears to inhibit Ca^{2+}-dependent secretion of AVP and OT via an A_1 receptor. This is in contrast with previous results that ATP can stimulate Ca^{2+}-dependent secretion of neurotransmitters/ neurohormones from neurons or endocrine secretory cells (Evans *et al.* 1992; Scanziani *et al.* 1992; Zimmermann, 1994; Troadec *et al.* 1998), but could explain why ATP appeared to inhibit AVP release from intact neurohypophyses (Sperlagh *et al.* 1999).

In fact, the effects of ATP and adenosine are not always easily distinguished. Added to a tissue preparation, ATP is hydrolysed by ecto-nucleotidases and, thus, might act via any of the metabolites formed. ATP released from a tissue might be hydrolysed to adenosine before it appeared in the perfusate and thus ATP effects might be mistaken for those of adenosine (El-Monatassim *et al.* 1992; Zimmermann, 1994). In the case of the NH nerve terminals, the surface-located ecto-nucleotidases, including ecto-ATPase, ecto-ADPase and ecto-5′nucleotidase, responsible for hydrolysis of ATP have been found at sites of ATP release (Thirion *et al.* 1996). The end product of ATP hydrolysis is generally adenosine, which is also a signalling substance (Burnstock, 1978). The fact that adenosine is a more potent (with an IC_{50} of 0.87 μM) inhibitor of the transient Ba^{2+} current than its precursor, ATP (see Figs. 1*A* and 6*B*), indicates that this nucleotide could inhibit the Ba^{2+} current via its metabolite.

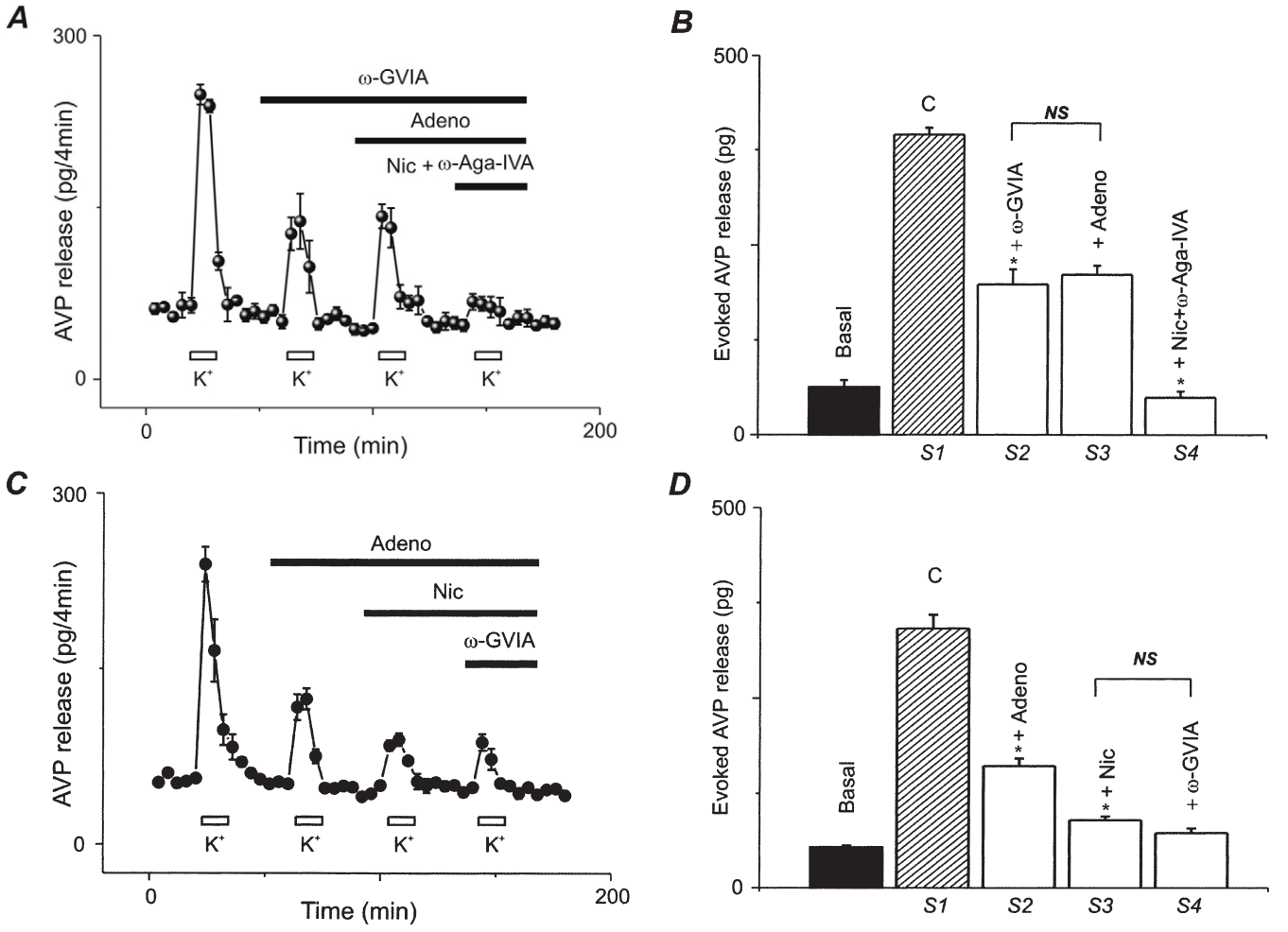

Figure 5. Effect of Ca^{2+} channel blockers and adenosine on AVP release

A, nerve terminals were challenged for 8 min with 50 mM K^+ repeated at four regular time intervals either in the absence (S1) or in the presence of 800 nM ω-conotoxin GVIA (S2), plus 200 μM adenosine (S3), plus 1 μM nicardipine and 450 nM of ω-AgaIVA (S4) as indicated by the bars above the data points. All blockers were present for at least 16 min before, during, and after each K^+ challenge. *C*, similar experiments were performed, but by applying first adenosine (S2), followed by addition of nicardipine (S3) and then ω-conotoxin GVIA (S4). All drugs were present for at least 16 min before, during and after each high K^+ challenge. All traces represent the mean of 4–6 experiments ± S.E.M. *B* and *D*, bar graphs summarizing basal and evoked release for *A* and *C* expressed as means ± S.E.M., n = 4–6.

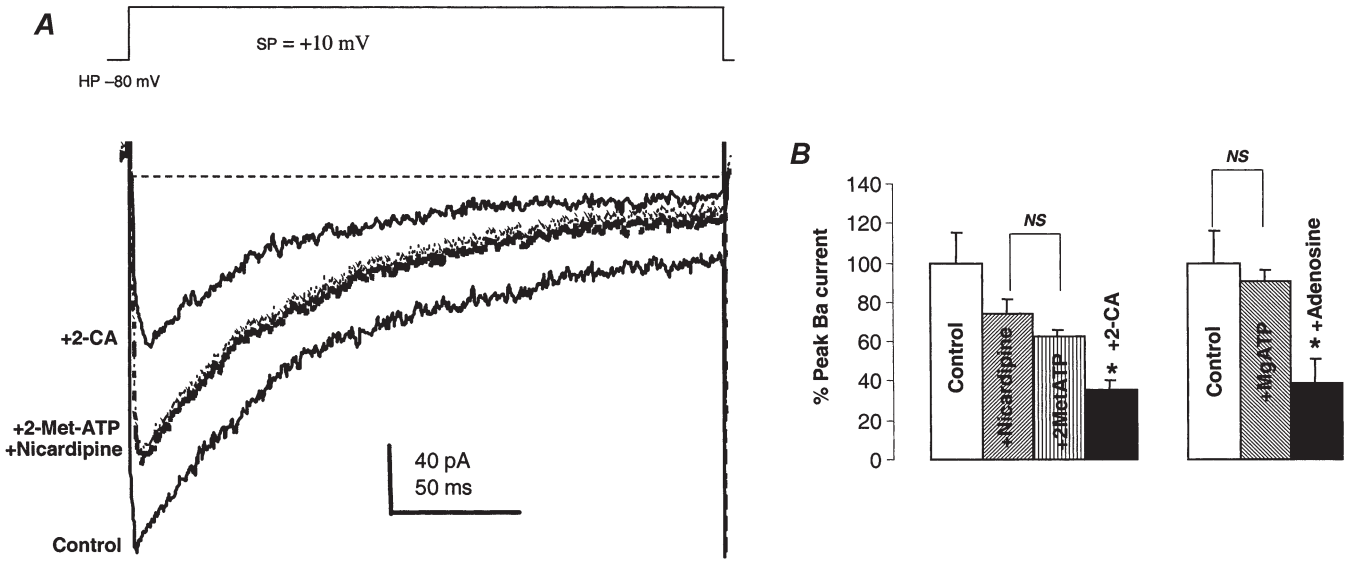

Figure 6. Only A_1 receptor agonists inhibit the N-type Ca^{2+} current in these nerve terminals

A, representative traces of Ba^{2+} currents (control) from a single isolated nerve terminal demonstrate that the long-lasting component of the terminal current is partially blocked by a saturating concentration (2.5 μM) of nicardipine. The purinergic (P_1) receptor agonist 2Met-ATP (at 20 μM) had no significant effect on the nicardipine-resistant Ba^{2+} current, but 20 μM 2-CA did inhibit it. The remaining current was due to the Q-type Ca^{2+} channel, since it was blocked by 100 μM ω-MVIIC (data not shown). *B,* statistical summary of the effects illustrated in *A* on the macroscopic Ba^{2+} currents. Only 2-CA shows a significant inhibition of the nicardipine-resistant transient Ba^{2+} currents in NH terminals. Furthermore, 20 μM adenosine had a significant effect on the current, whereas 1 mM Mg-ATP did not.

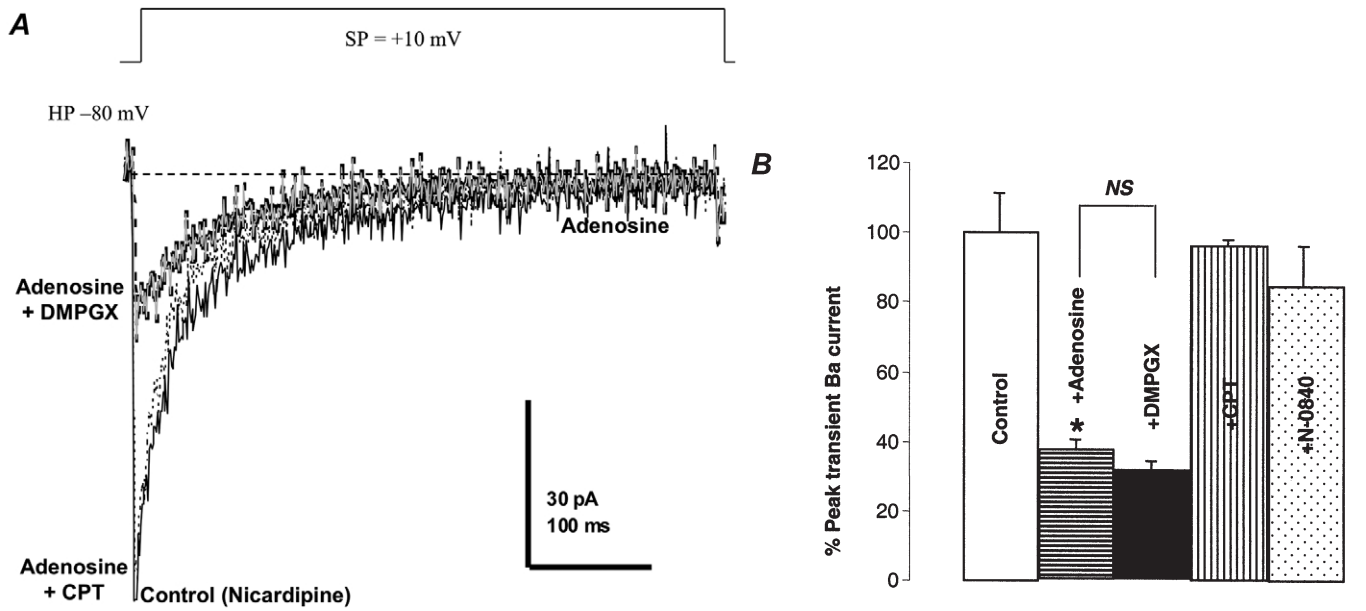

Figure 7. The Ba^{2+} currents inhibited by adenosine could be reversed by an A_1, but not an A_2, antagonist

A, the whole-terminal Ba^{2+} currents were elicited from −80 to +10 mV in the presence of 2.5 μM nicardipine (control trace). Adenosine (20 μM) was applied to inhibit the Ba^{2+} current (black, dashed trace). Addition of 20 μM DMPGX, an A_2 receptor antagonist, could not reverse the inhibition by adenosine of the transient current (grey trace). However, further addition of 20 μM CPT, an A_1 receptor antagonist, almost completely reversed this inhibition (dotted line). *B*, statistical summary of the effects illustrated in *A* on the macroscopic Ba^{2+} currents. Only CPT shows a significant inhibition of the inhibition by adenosine of transient Ba^{2+} currents in NH terminals. Furthermore, 20 μM N-0840, another A_1 antagonist, had similar effects.

Burnstock (1978) was the first to suggest a sub-classification of purinoceptors into two classes: P_1 purinoceptors (including A_1 and A_2 adenosine receptors) with an agonist potency order of adenosine > AMP > ADP > ATP and P_2 purinoceptor with a reverse ranking: ATP > ADP > AMP > adenosine. The use of different agonists for these purinergic receptors, some of which are non-hydrolysable, allowed us to distinguish ATP effects from those of adenosine.

Judging from the different potency of adenosine, 2-CA, ATP and 2Met-ATP on the N-type Ca^{2+} channel currents (Figs 1, 2 and 6), our data strongly suggest that adenosine exerted its reversible effects on the N-type channel through the activation of an A_1 purinoceptor on the terminal membrane. Our results obtained from the secretory terminals of NH are in agreement with those found in other neurotransmitter systems, in that adenosine specifically blocks pre-synaptic voltage-gated N-type Ca^{2+} channels (Yawo & Chuhma, 1993). ATP, however, might also act through another pathway in this model system, which may be coupled to the inhibition of the $K_{Ca^{2+}}$ channel on the terminal membrane (Wang *et al.* 1992*a*; Lemos & Wang, 2000). Most importantly, the antagonist data also indicate that the activation of the A_1 purinoceptor mediates the adenosine Ca^{2+} channel block (Fig. 7) and the subsequent inhibition of AVP release (Fig. 8).

Furthermore, it appears that adenosine specifically blocks the transient N-, instead of the Q-, R- or L-type Ca^{2+} channels in the NH nerve terminals through the activation of A_1 adenosine receptors. The exact mechanism by which the signal transduction pathway of cellular responses of NH nerve terminals to extracellular adenosine occurs,

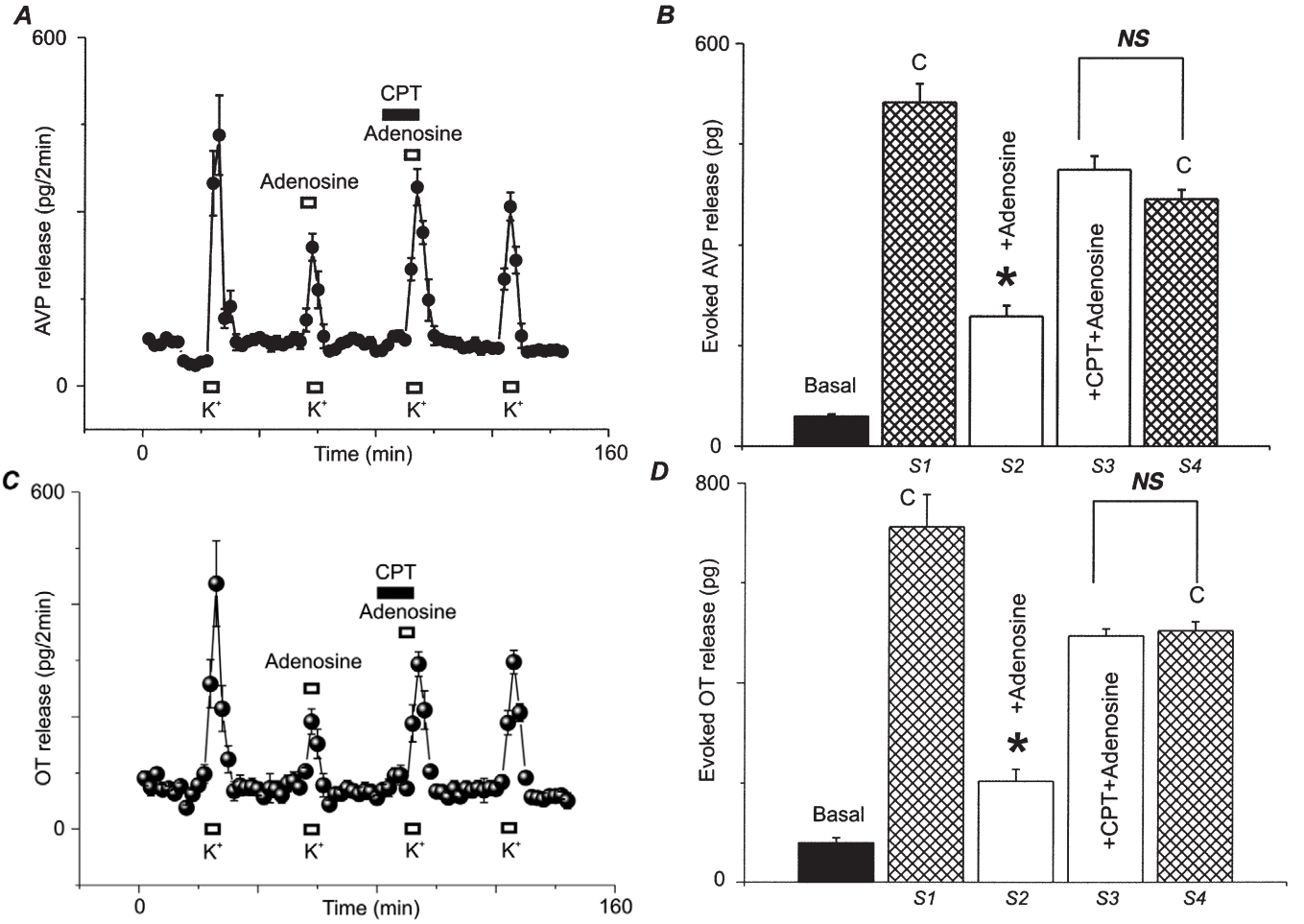

Figure 8. Effects of adenosine on the release of AVP and OT from isolated rat NH nerve terminals could be reversed by an A_1 antagonist

AVP (*A*) and OT (*C*) release was stimulated (S) for 4 min by 50 mM KCl (K^+) repeated four times (S1–4) at regular time intervals. Nerve terminals were challenged with high K^+ either in the absence (S1, S4) or in the presence (S2, S3) of 20 μM adenosine. Preincubation with 20 μM CPT, an A_1 receptor antagonist, almost completely reversed the inhibition by adenosine (S3). All drugs were present as indicated by bars above each high K^+ challenge. *B* and *D*, basal and evoked release summarized for AVP and OT, respectively.

however, is still unknown. Nevertheless, in other neuronal systems, activation of purinergic receptors similarly induces an inhibition of neurotransmitter release by pre-synaptic mechanisms, predominantly by blockade of N-type Ca^{2+} channels via activation of A_1 receptors (Yawo & Chuhma, 1993; Mogul *et al.* 1993; Dittman & Regehr, 1996; Ambrosio *et al.* 1997; Wu & Saggau, 1997). The mechanisms underlying the inhibition of N-type Ca^{2+} channels in pre-synaptic nerve terminals by adenosine are associated with a second message system, in which a pertussis toxin-sensitive G-protein (Dolphin & Prestwich, 1985) and protein kinase C (Budd & Nicholls, 1995) may be involved. In contrast to the A_1 receptor at pre-synaptic sites, activation of A_2 receptor enhances the evoked release of neurotransmitters or hormones (Popoli *et al.* 1995; Ambrosio *et al.* 1997; Kumari *et al.* 1999; Okada *et al.* 2001) that may involve P/Q-type Ca^{2+} channels at pre-synaptic sites (Mogul *et al.* 1993; Umemiya & Berger, 1994).

ATP directly blocks Ca^{2+}-activated K^+ channels in the nerve terminal (Lemos & Wang, 2000) and, via a P2X receptor, also increases intraterminal $[Ca^{2+}]_i$ (Troadec *et al.* 1998). These two effects appear to be additive, since the delay in repolarization of the action potential would also result in higher Ca^{2+} levels in the terminal and, thus, an increase in AVP release, in particular. As shown here, however, any subsequent inhibition is due to the hydrolysed metabolite of ATP, adenosine, which strongly and preferentially inhibits the N-type Ca^{2+} channel, leading to a decrease in both AVP and OT release.

In conclusion, purinergic modulation of these nerve terminal ion channels, even though complicated by its multiple effects, is physiologically significant. We hypothesize that during depolarization–secretion coupling in the NH terminals, ATP, co-released with the hormones, would initially act locally to block K_{Ca} channels (Lemos & Wang, 2000). This would result in a prolongation of action potentials and, via P2X2 receptors, open divalent cation channels (Troadec *et al.* 1998), both leading to more subsequent Ca^{2+} entry, and thus, more AVP release. ATP would, meanwhile, be rapidly hydrolysed to adenosine by ecto-nucleotidases bound to the pre-synaptic membrane (Thirion *et al.* 1996). Adenosine could then act through the A_1 receptor to specifically block the N-type Ca^{2+} channel and decrease the release of both neuropeptides. These dual, conflicting autocrine/paracrine effects of ATP released from NH terminals and its metabolite, adenosine, could help explain (see also Lemos & Wang, 2000) why the levels of AVP *vs.* OT release are optimized as a result of different physiological patterns of stimulation *in vivo* (Cazalis *et al.* 1985). These multiple feedback effects could also have widespread ramifications in terms of CNS synaptic modulation.

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