British Journal of Pharmacology (2001) 133, 337–344 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00

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Inhibition of calcium channels by opioid- and adenosine-receptor agonists in neurons of the nucleus accumbens

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1 The pharmacological effects of opioid- and adenosine-receptor agonists on neural signalling were investigated by measuring drug actions on barium current flowing through calcium channels in acutely-dissociated neurons of the rat nucleus accumbens (NAc).

2 Under whole-cell voltage clamp, opioids acted *via* μ, but not δ or κ, receptors to partially inhibit barium current. Mean inhibition was $35 \pm 2\%$ ($\pm$s.e.mean, $n=33$) for methionine-enkephalin and $37 \pm 1\%$ ($n=65$) for the selective μ receptor agonist DAMGO, both measured at saturating agonist concentrations in neurons with diameter $\geqslant 20$ μm. EC_{50} for DAMGO was 100 nM. Perfusion of naloxone reversed the current inhibition by DAMGO.

3 Adenosine also partially inhibited barium current in these neurons. Mean inhibition was $28 \pm 2\%$ ($n=29$) for adenosine and $33 \pm 3\%$ ($n=27$) for the selective A_1 receptor agonist N^6CPA, both at saturating concentrations in neurons with diameter $\geqslant 20$ μm. EC_{50} for N^6CPA was 34 nM. Adenosine inhibition was reversed by perfusion of an A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine, while the selective A_{2A} receptor agonist, CGS 21680, had no effect.

4 Inhibition by opioids and adenosine was mutually occlusive, suggesting a converging pathway onto calcium channels.

5 These actions involved a G-protein-coupled mechanism, as demonstrated by the partial relief of inhibition by strong depolarization and by the application of N-ethylmaleimide or GTP-γ-S.

6 Inhibition of barium current by opioids had their greatest effect in large neurons, that is, in presumed interneurons. In contrast, opioid inhibition in neurons with diameter $\leqslant 15$ μm was $11 \pm 2\%$ ($n=26$) for methionine-enkephalin and $11 \pm 4\%$ ($n=17$) for DAMGO, both measured at saturating agonist concentrations. Adenosine inhibition in neurons with diameter $\leqslant 15$ μm was $22 \pm 5\%$ ($n=9$).

7 These results implicate the interneurons as a locus for the modulation of the excitability of projection neurons in the NAc during the processes of addiction and withdrawal.

British Journal of Pharmacology (2001) **133,** 337–344

Keywords: Opioid; DAMGO; nucleus accumbens; calcium channel; opioid withdrawal; interneuron; adenosine

Abbreviations: aCSF, artificial cerebrospinal fluid; DAMGO, [D-ala^2-NMePhe^4, Gly-ol]-enkephalin; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate); NAc, nucleus accumbens; N^6CPA, N^6-cyclopentyladenosine; NEM, N-ethylmaleimide; VTA, ventral tegmental area

Introduction

The nucleus accumbens (NAc), together with the midbrain ventral tegmental area (VTA), forms the core of the mesolimbic dopaminergic system which is responsible for the behavioural manifestations of opioid addiction, such as reward, sensitization, craving and withdrawal (Self & Nestler, 1995). The output neurons of NAc are the GABAergic spiny projection neurons, which are thought to be regulated by both afferent inputs (e.g. from prefrontal cortex and VTA) and interneurons within the NAc (Wilson, 1998). By altering the excitability of spiny projection neurons, these two classes of synaptic input could have a profound effect on the process of addiction.

Symptoms of opioid withdrawal, which occur following the removal of agonist from opioid receptors, can be alleviated by re-administration of opioids. Adenosine receptor agonists have also been reported to alleviate opioid withdrawal in dependent animals, while adenosine antagonists exacerbate withdrawal (Dionyssopoulos *et al*., 1992; Kaplan & Sears, 1996; Salem & Hope, 1997). Furthermore, acute administration of adenosine receptor antagonists to naive animals has been found to mimic opioid withdrawal (Collier *et al*., 1974). Thus, it is evident that adenosine receptors play a significant role in the manifestation of opioid withdrawal symptoms. The mechanism underlying this interaction between opioid and adenosine receptors remains unclear.

The modulatory actions of opioid- and adenosine-receptor agonists have been studied in a number of *in vitro* preparations. At the cellular level, both have been shown to inhibit adenylyl cyclase and increase potassium conductance in regions of the brain important for addictive behaviour (Dhawan *et al*., 1996; Ralevic & Burnstock, 1998). Opioids and adenosine have also been found to inhibit release of

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GABA and glutamate from presynaptic terminals in the NAc (Chieng & Williams, 1998; Manzoni *et al*., 1998; Yuan *et al*., 1992).

Here we report a novel inhibitory effect of opioid- and adenosine-receptor agonists in acutely-dissociated neurons of the NAc. Both classes of agonist inhibited calcium channels *via* a converging G-protein-mediated mechanism, with a profound inhibition in all large cells, i.e. the presumed interneurons. These results suggest a possible cellular locus for the interaction between opioid and adenosine receptors during withdrawal.

Methods

Cell dissociation

Acutely dissociated neurons from the NAc were prepared from Wistar rat pups (14–21 days old) using procedures that were approved by the Animal Experimentation Ethics Committee of the Australian National University. Animals were killed under deep halothane anaesthesia and horizontal forebrain slices (350 μm) were cut in chilled physiological saline (4°C) using a vibratome. The shell of NAc at the level of the anterior commissure was identified using a rat brain atlas (Paxinos & Watson, 1986) and micro-punched out from the slices. Tissue pieces were incubated in oxygenated enzyme solution containing papain (10 units ml^{-1}) for 1 h at 35°C. The tissue was triturated to dissociate individual neurons, which were placed in a dish and allowed to settle for at least 30 min before being used for physiological recordings. Slice preparation and dissociation were done in a low calcium (0.1 mM) Earle's Balanced Salt solution supplemented with 1 mM kynurenic acid to block glutamate receptors. For the experiments which involved measurement of a hyperpolarization-activated cation current, I_h, the enzymatic incubation step was omitted.

Electrophysiological recordings

The NAc neurons were visualized with an inverted microscope and whole-cell recordings were made at room temperature (22–25°C) using a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA, U.S.A.). The external solution for measuring barium currents through calcium channels comprised (in mM): tetraethylammonium chloride 145, $BaCl_2$ 6, CsCl 2.5, HEPES 10, glucose 10, pH 7.3. Patch electrodes (3–5 MΩ) contained intracellular solution comprising (in mM): CsCl 110, $CaCl_2$ 2, EGTA 10, Mg-ATP 5, Na-GTP 0.2, HEPES 10, pH 7.3. Acceptable access resistance was <20 MΩ, periodically monitored with repetitive 10 mV steps. Series resistance compensation of 80% was used for all experiments. Families of barium currents, evoked by a sequence of test pulses (−60 to +60 mV), were acquired every 30 s. Test pulses were preceded by a 5 ms-long prepulse to −90 mV to remove resting inactivation (Figure 1a; Chieng & Bekkers, 1999). Linear leak currents and capacitance transients were subtracted using a P/4 protocol. Cells were maintained in constantly-flowing external solution, delivered through a series of identical glass flow pipes. Drugs were dissolved in external solution and applied *via* these pipes. For measuring

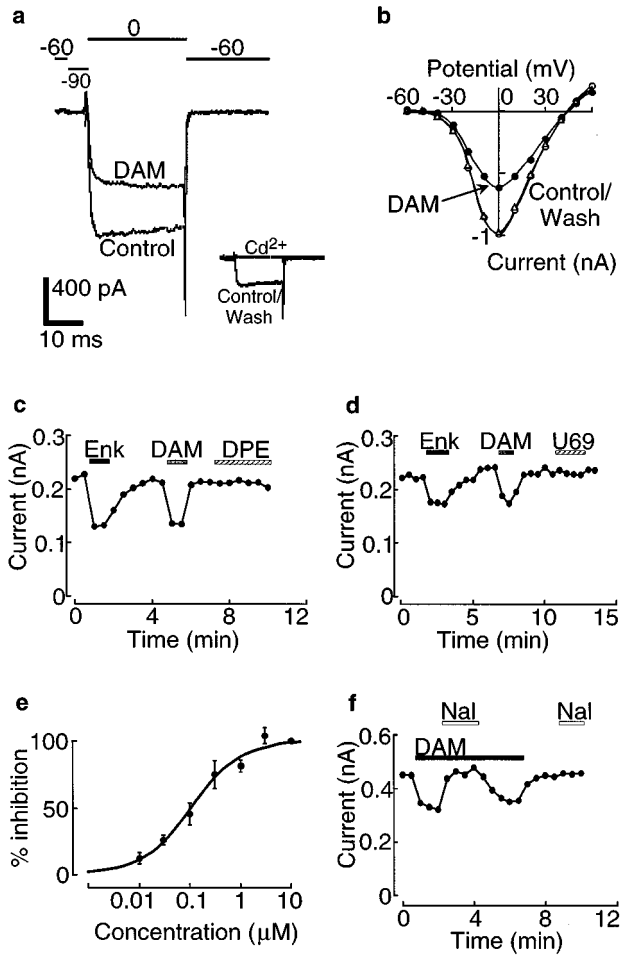

Figure 1 Opioids act *via* μ receptors to inhibit barium current flowing through calcium channels in acutely-dissociated NAc neurons. (a) Example of barium current recorded during a test pulse to 0 mV from a pre-pulse of −90 mV (Control). This current was partially blocked by perfusion of selective μ agonist DAMGO (DAM, 10 μM) and was fully blocked by cadmium (Cd^{2+}, 100 μM, inset). The pulse protocol is shown above. Linear leak currents and capacitance transients have been subtracted using a P/4 protocol. (b) Current-voltage plot of barium current measured in one cell (same as in a). (c) Plot of amplitude of barium current at 0 mV *versus* time during the experiment. Horizontal bars indicate periods of perfusion with methionine-enkephalin (Enk, 100 μM), DAMGO (DAM, 10 μM) and selective δ agonist DPDPE (DPE, 3 μM). (d) Inhibition of current by methionine-enkephalin (Enk, 100 μM) and DAMGO (DAM, 10 μM) but not selective κ agonist U69593 (U69, 10 μM). (e) Plot showing the inhibition of barium current as a function of DAMGO concentration. The ordinate is expressed as a percentage of maximal inhibition (at 10 μM). Each point is an average of six cells (±s.e.mean). The smooth curve is a fit of a logistic function, giving an EC_{50} of 100 nM. (f) Inhibition of barium current by DAMGO (DAM, 1 μM) was reversed by naloxone (Nal, 1 μM).

I_h, the external solution was a high potassium artificial cerebrospinal fluid (aCSF) with composition (mM): NaCl 126, KCl 12.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11, $NaHCO_3$ 24, gassed with 95% O_2/5% CO_2. Data was acquired using pClamp 6 and analysed with AxoGraph 4.0 (Axon Instruments). The amplitude of I_{Ba} was found by averaging over a 15 ms-long time window at the end of the test pulse. Drug timecourse plots (e.g. Figure 1c) show the amplitude of I_{Ba} measured at the peak of the current-voltage

plot (i.e. test pulse to −10 or 0 mV). Inhibition was expressed as a percentage of the current amplitude measured just before drug application. Sample means were statistically compared using the *t*-test. Errors are given as ±s.e.mean.

Drugs

Adenosine, [D-ala^2-NMePhe^4, Gly-ol]-enkephalin (DAMGO), [D-Pen^2, D-Pen^5]-enkephalin (DPDPE), guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S), kynurenic acid, methionine-enkephalin, N-ethylmaleimide (NEM), papain and tetraethylammonium chloride were from Sigma (St Louis, MO, U.S.A.). CGS 21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), naloxone, N^6-cyclopentyladenosine (N^{6}CPA) and U69593 were from Research Biochemicals (Natick, MA, U.S.A.).

Results

Standard whole-cell patch clamp techniques were used to measure barium current flowing through calcium channels in neurons acutely dissociated from the shell region of NAc. The current was reversibly abolished by perfusion of a calcium channel blocker, Cd^{2+} (100 μM, Figure 1a inset). Cell types were categorized according to pharmacology and size of soma (e.g. Figure 4). Large cells (soma diameter ⩾20 μm) were most likely to be interneurons because all projection neurons are smaller (mean diameter ∼15 μm; Kawaguchi, 1997; Wilson, 1998). It was noted that opioid receptor agonists produced a consistently greater effect in the larger cells (Figure 4; see later). Unless otherwise stated, the pharmacology experiments reported here were taken from the large-neuron group.

Opioids inhibit calcium channels in NAc

The μ opioid receptor agonist, DAMGO (1–10 μM), partially inhibited barium current without shifting the current-voltage relationship along the voltage axis (mean inhibition 37±1%, $n=65$, significantly different from control current, $P<0.05$; Figure 1a,b). This action of DAMGO was mimicked by another opioid receptor agonist, methionine-enkephalin (10–100 μM, 35±2%, $n=33$, not significantly different from DAMGO inhibition, $P>0.05$), but not by selective δ and κ receptor agonists DPDPE (1 μM, 3±2%, $n=8$, not significantly different from control current, $P>0.05$) and U69593 (10 μM, 5±2%, $n=11$, $P>0.05$), respectively (Figure 1c,d). These results suggest a specific μ opioid receptor-mediated effect. Inhibition by DAMGO had an estimated EC_{50} of 100 nM (Figure 1e) and was reversibly blocked by co-perfusion of an opioid receptor antagonist (naloxone, 1 μM, residual inhibition 1±3%, $n=5$, $P>0.05$ *cf.* pre-drug baseline; Figure 1f). In the same neurons, inhibition by DAMGO was 32±3% ($n=5$). Application of naloxone by itself had no effect ($n=5$, $P>0.05$; Figure 1f).

Opioid and adenosine inhibition of calcium channels is mutually occlusive

Like opioids, adenosine partially inhibited barium current flowing through calcium channels (100–300 μM, 28±2%, $n=29$). This adenosine-mediated inhibition was mimicked by perfusion of an A_1 receptor agonist, N^{6}CPA (1–10 μM, 33±3%, $n=27$, not significantly different from adenosine inhibition, $P>0.05$), and was reversed by co-application with an A_1 receptor antagonist, DPCPX (1 μM, residual inhibition 2±2%, $n=3$, $P>0.05$ *cf.* pre-drug baseline), suggesting an adenosine A_1 receptor-mediated action (Figure 2a,c). In these same neurons, inhibition by adenosine was 29±3% ($n=7$). Inhibition by N^{6}CPA had an estimated EC_{50} of 34 nM (Figure 2b). In the presence of adenosine or N^{6}CPA, the current-voltage relationship was not shifted along the voltage axis (not illustrated). The selective adenosine A_{2A} receptor agonist, CGS 21680 (1 μM), was ineffective in inhibiting barium current (4±2%, $n=7$, $P>0.05$ *cf.* pre-drug baseline; Figure 2d). In these same cells, inhibition by adenosine was 29±3% ($n=7$).

Co-application of saturating concentrations of DAMGO (10 μM) and adenosine (200 μM; see Manzoni *et al.*, 1998) produced little further inhibition beyond that produced by each drug alone in the same cells, regardless of the order of application (29±6% for adenosine alone; 36±2% for adenosine plus DAMGO; 32±4% for DAMGO alone; 35±6% for DAMGO plus adenosine; $n=5$, $P>0.05$; Figure 2e,f). This suggests opioid and adenosine receptors activate a converging pathway leading to the inhibition of calcium channels.

G-protein involvement in opioid and adenosine inhibition of calcium channels

The inhibitory actions of opioid- and adenosine-receptor agonists in other systems have been reported to occur *via* a G-protein mediated pathway (Dhawan *et al.*, 1996; Ralevic & Burnstock, 1998). Here, we noted a moderate amount of slowing in the activation kinetics of barium current by opioids and adenosine, suggesting a possible involvement of G-proteins (e.g. Figures 1a and 2a; Hille, 1994). In the large cells, opioids significantly prolonged the 5–95% risetime of the activation of barium current (methionine-enkephalin, 10–100 μM, 3.8±0.4 ms before *versus* 7.8±0.7 ms after, $n=33$, $P<0.05$; DAMGO, 1–10 μM, 3.1±0.2 ms before *versus* 6.1±0.5 ms after, $n=65$, $P<0.05$). Adenosine and N^{6}CPA also increased the 5–95% risetime (adenosine, 100–300 μM, 3.1±0.2 ms before *versus* 4.3±0.5 ms after, $n=29$, $P<0.05$; N^{6}CPA, 1–10 μM, 2.7±0.2 ms before *versus* 4.7±0.8 ms after, $n=27$, $P<0.05$).

Involvement of a G-protein-mediated process was further supported by the observation that strong depolarization of neurons prior to activation of barium current significantly relieved the opioid inhibition (DAMGO, 10 μM, 43±3% before *versus* 24±3% after strong depolarization, $n=6$, $P<0.05$; Figure 3b–d). A similar effect was found for adenosine (200 μM, 37±4% before *versus* 18±2% after, $n=7$, $P<0.05$; Figure 3a,c,d). Note that strong depolarization alone increased the amplitude of the barium current in these neurons (e.g. Figure 3a; compare traces 1 and 3), suggesting that tonic inhibition was present before application of the agonists (Dolphin, 1998).

Prior exposure of neurons to a G-protein inhibitor, NEM (50–100 μM for 2–3 min), also significantly reduced opioid inhibition of the barium current (pre-NEM, 35±4%; post-NEM, 12±2%; $n=8$, $P<0.05$; Figure 3d–f). Addition of GTP-γ-S (400 μM) to the pipette solution produced a gradual

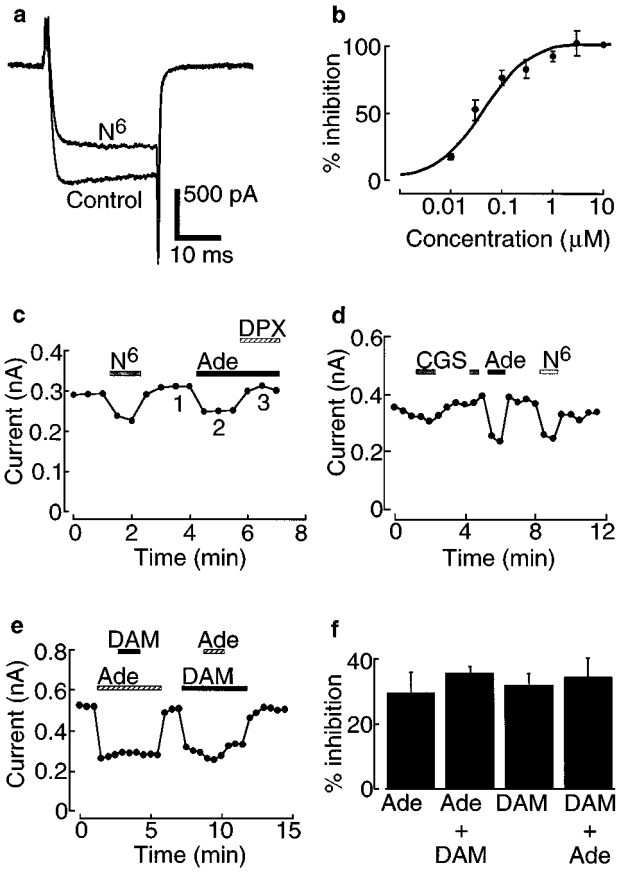

Figure 2 Adenosine acts *via* A_1 receptors to inhibit barium current and is mutually occlusive with opioids. (a) Example of barium current inhibition by the A_1 receptor agonist, N^6CPA (N^6, 1 μM). (b) Concentration-response curve of N^6CPA with the ordinate expressed as a percentage of maximal inhibition (at 10 μM). Each point is an average of 5–11 cells (±s.e.mean). The smooth curve is a fit of a logistic function, giving an EC_{50} of 34 nM. (c) Timecourse plot showing that N^6CPA (N^6, 1 μM) mimicked the action of adenosine (Ade, 10 μM). Perfusion of the selective A_1 receptor antagonist, DPCPX (DPX, 1 μM), reversed the inhibition caused by adenosine. (d) The selective A_{2A} receptor agonist, CGS 21680 (CGS, 1 μM), did not inhibit barium current. Adenosine and N^6CPA concentrations were 200 μM and 1 μM, respectively. (e) Timecourse plot showing that saturating concentrations of adenosine (Ade, 200 μM) and DAMGO (DAM, 10 μM) applied together did not produce greater inhibition than did each drug applied alone. (f) Summary of the results of five experiments as in (e).

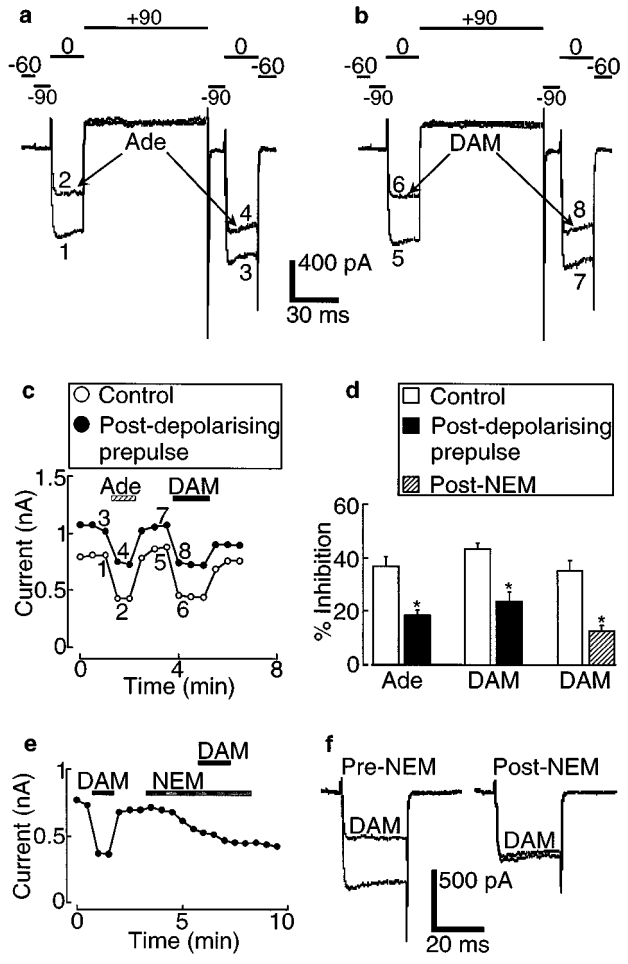

Figure 3 Inhibition of barium current by opioids and adenosine is mediated by a G-protein-coupled pathway. (a and b) Strong depolarization partially relieved inhibition of barium current by adenosine (Ade, 200 μM) and DAMGO (DAM, 10 μM). Amount of inhibition by each drug (step from −90 to 0 mV) was compared before (e.g. numbers 1 and 2) and after (e.g. numbers 3 and 4) application of the strong depolarising prepulse to +90 mV. The pulse protocol is shown at the top. Numbers 1–8 identify the time points in (c). (c) Timecourse plot for the same cell as shown in (a) and (b). Symbols represent the amplitude of the barium current averaged over a 15 ms-long window at the end of the test pulse to 0 mV. (d) Pairs of bars on the left and centre summarise the experiments in (a)–(c) for adenosine and DAMGO. These show the mean inhibition of barium current by adenosine (Ade; $n=7$) or DAMGO (DAM; $n=6$) before (open bars) and after (filled bars) the strong depolarization. Pair of bars on the right summarises a different experiment in which inhibition by DAMGO was measured before and after perfusion with N-ethylmaleimide (NEM, 50 or 100 μM for 2–3 min; $n=8$). An example is shown in (e) and (f). Statistically significant differences are indicated by the asterisks ($P<0.05$). (e) NEM (100 μM) reduced inhibition of barium current by DAMGO (DAM, 1 μM). (f) Raw data traces from the same cell as in (e), before and after application of NEM.

slowing of the current kinetics and a reduction in the amplitude, consistent with a baseline turnover of G-proteins in these cells. After the current had stabilized (15 min), DAMGO (1–10 μM) no longer inhibited the current (3±2%, $n=4$, $P<0.05$ *cf.* control inhibition). All these findings point to the involvement of G-proteins in the inhibitory actions studied here.

Opioids exert greater effects in large neurons

Opioids reversibly inhibited barium current in all large cells (soma diameter ⩾20 μm; Figure 4a,c). At saturating agonist concentrations, the mean inhibition in these cells was 35±2% ($n=33$; open circles, Figure 4a) for 10–100 μM methionine-enkephalin and 37±1% ($n=65$; closed circles, Figure 4a) for 1–10 μM DAMGO. Methionine-enkephalin and DAMGO also inhibited barium current in a proportion of smaller neurons (diameter ⩽15 μm) but the effect was significantly less than the inhibition in large cells (Figure 4a,c): mean inhibition in small cells was 11±2% ($n=26$, $P<0.05$ *cf.* large cells) for methionine-enkephalin, and 11±4% ($n=17$, $P<0.05$) for DAMGO.

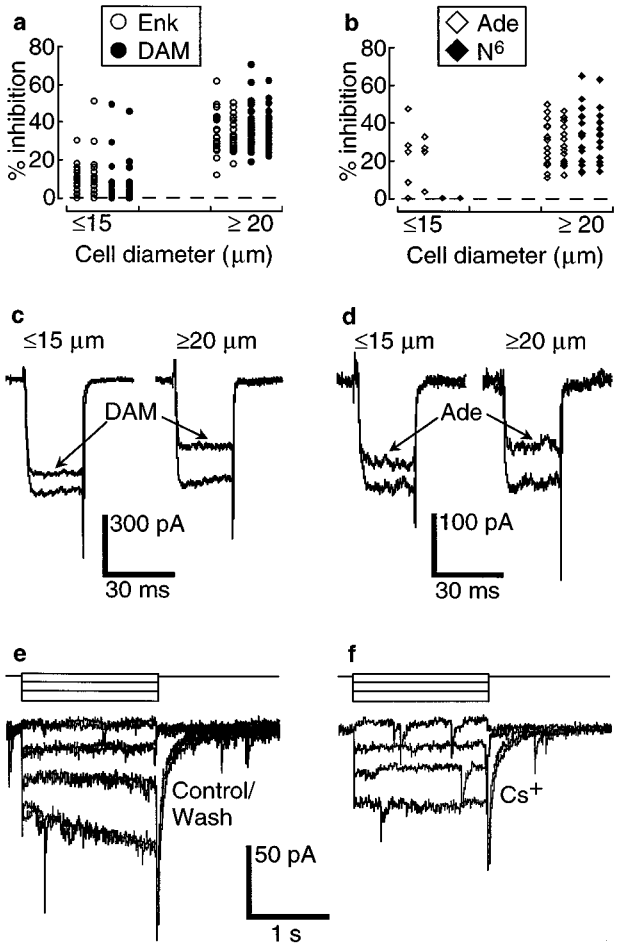

Figure 4 Opioid-receptor agonists preferentially inhibit barium current in large cells. (a) Per cent inhibition of barium current by opioids (methionine-enkephalin, 10–100 μM; DAMGO, 1–10 μM) in individual neurons, sorted by soma diameter. Each circle represents a single neuron, grouped into columns for clarity. For mean inhibition values, see the text. (b) A similar plot for adenosine (100–300 μM) and N^6CPA (1–10 μM). (c and d) Representative examples of the different categories of experiment shown in (a) and (b). (e) Examples of I_h recorded in a large ($\geq$20 μm diameter) cell. The cell was held at -50 mV and stepped to -40, -70, -100 and -130 mV (protocol at top). (f) Bathing with 2 mM Cs^+ blocked I_h; control and wash traces overlay in (e). Note the spontaneous synaptic activity, commonly seen when cells were dissociated without using enzymes, as for this experiment.

Like opioids, adenosine and N^6CPA inhibited barium current in all large cells: the mean inhibition was $28\pm2\%$ ($n=29$; open diamonds, Figure 4b) for 100–300 μM adenosine and $33\pm3\%$ ($n=27$; closed diamonds, Figure 4b) for 1–10 μM N^6CPA. In neurons ≤15 μm in diameter, adenosine (100–300 μM) inhibited barium current by $22\pm5\%$ ($n=9$, $P>0.05$ *cf.* large cells) and N^6CPA (10 μM) had no effect in two cells tested (Figure 4b,d).

Large and small cells also differed in their expression of a hyperpolarization-activated current, I_h. When the membrane potential was stepped to very negative values (-130 mV) in a high potassium aCSF (Methods), large cells exhibited I_h ($n=6$) which could be blocked by external application of Cs^+ (2 mM; Figure 4e,f), whereas small cells contained no I_h ($n=4$; not illustrated). Large cells were predominantly bipolar in shape, 59/85 or 69%. The rest were made up of triangular (25%) and multipolar (6%) cells. Small cells were primarily round or bipolar in shape (29/33 or 88%) while the remainder were triangular (6%) and multipolar (6%).

Discussion

These results indicate that opioid- and adenosine-receptor agonists act *via* a converging G-protein-mediated pathway to inhibit voltage-activated calcium channels in single neurons of the NAc. The larger cells were more sensitive to opioid inhibition. In the neostriatum, most larger neurons in the NAc are cholinergic interneurons which express I_h (Kawaguchi, 1997). Although we did not check the neurochemical identity of the large cells in our data set, many of those tested for I_h did contain this current (Figure 4e), suggesting that they are likely to be cholinergic interneurons. The smaller cells likely comprise a heterogeneous population of spiny projection neurons and small interneurons (Kawaguchi, 1997; Wilson, 1998), which is probably reflected in the highly variable inhibition seen in cells within this group (Figure 4a,b). In the absence of clear criteria for distinguishing the different types of smaller cells, they were not further examined here.

Modulation of excitability of neurons within the NAc

Opioid- and adenosine-receptor agonists have been reported to produce modulatory actions in a wide variety of preparations. In regions that are crucial to opioid addiction, such as the NAc, VTA and the midbrain periaqueductal grey, opioids and adenosine inhibit presynaptic release of GABA and glutamate, and increase postsynaptic potassium conductance (Bagley *et al*., 1999; Bonci & Williams, 1996; Chieng & Christie, 1994a,b; Chieng & Williams, 1998; Johnson & North, 1992; Manzoni & Williams, 1999; Shoji *et al*., 1999; Uchimura & North, 1991; Yuan *et al*., 1992). Here we report that opioids and adenosine also inhibit calcium channels in neurons dissociated from the shell of NAc. The shell region of NAc was selected in the present study because the shell but not the core of NAc is thought to be involved in addiction to substances of abuse (Carlezon & Wise, 1996; Mcbride *et al*., 1999).

The consequences of this inhibition for the excitability of NAc neurons will depend upon the function of the targeted calcium channels. For example, if these channels participate in burst firing, as in some pyramidal cells (Williams & Stuart, 1999), their inhibition will dampen postsynaptic excitability. If the targeted calcium channels trigger the synaptic release of neurotransmitter, their inhibition will depress synaptic transmission. Alternatively, inhibition of calcium entry may reduce calcium-activated potassium conductances, increasing neuronal excitability. Thus, the net effect of opioids and adenosine on individual NAc neurons is likely to be complicated, and the consequences for NAc as a whole will depend upon the balance of synaptic excitation and inhibition within this nucleus.

Pharmacology of the inhibition

The opioid effect was entirely mediated *via* μ receptors (Figure 1). This is somewhat surprising, since prominent

actions of δ and κ receptors in the NAc have been reported in a number of studies (e.g. Heijna *et al*., 1992; Tjon *et al*., 1995). The specificity of the effect seen here may be due in part to sampling bias (recordings were preferentially obtained from large cells) or to the loss of distal dendrites and axons following mechanical dissociation. Nevertheless, the opioid effects in our preparation exhibited classic μ receptor pharmacology, similar to that in other systems (e.g. Chieng & Christie, 1994a; Christie *et al*., 1987). This was demonstrated both by the actions of subtype-selective agonists (Figure 1c,d), and by the EC_{50} for DAMGO of 100 nM (Figure 1e), which is similar to the value found for μ receptors elsewhere ($\sim$80 nM; Chieng & Christie, 1994a; Chieng & Williams, 1998). In the NAc, although labelling studies have demonstrated a presence of μ, δ and κ receptors or the mRNAs that encode them, correlation of different receptors to cell types has been lacking (Delfs *et al*., 1994; Georges *et al*., 1998; Svingos *et al*., 1997).

The adenosine action was mediated *via* adenosine A_1 receptors, because a selective A_1 receptor agonist mimicked the adenosine effect and a selective A_1 receptor antagonist abolished it (Figure 2c). The EC_{50} of N^6CPA obtained here (34 nM) was also similar to that found in other cell types (43 nM, Bonci & Williams, 1996; 63 nM, Manzoni *et al*., 1998). An A_1 receptor-mediated effect has been reported in the NAc, acting on postsynaptic potassium channels (Uchimura & North, 1991) as well as on presynaptic terminals (Chieng & Williams, 1998; Manzoni *et al*., 1998). According to immunohistochemical studies, the NAc contains adenosine A_1 receptors, but detailed work on matching the receptors with neuronal subtypes has not been done (Rivkees *et al*., 1995). The NAc is also strongly labelled for adenosine A_{2A} receptors (Johansson & Fredholm, 1995). The absence of an A_{2A} receptor-mediated effect in the present study may be due to our use of a dissociated cell preparation lacking synaptic terminals (Figure 2d; e.g. Kirk & Richardson, 1994).

Mechanism of the inhibition

Our results strongly implicate the involvement of a G-protein pathway in the inhibition of calcium channels by opioids and adenosine in the NAc. This is indicated by the slowing of the activation kinetics of the inhibited current (e.g. Figures 1a and 2a), the partial relief of agonist inhibition of current by prior strong depolarization (Figure 3a–d), and the block of inhibition by NEM (Figure 3d–f) and GTP-γ-S.

The slowing of activation kinetics is characteristic of a G-protein mediated action due to partial dissociation of G$\beta\gamma$ subunits from calcium channels (Dolphin, 1998; Ikeda, 1996). G$\beta\gamma$ has been shown in expression systems to produce a tonic inhibition of calcium currents which can be relieved by strong depolarization (Dolphin, 1998; Ikeda, 1996). Such inhibition is apparent for the control traces in Figure 3a,b (compare traces 1 with 3 and 5 with 7) suggesting that G$\beta\gamma$ may be tonically active in NAc interneurons. Application of adenosine and opioids presumably increases the levels of G$\beta\gamma$, further inhibiting calcium channels in a depolarization-sensitive manner (Figure 3a,b).

NEM is a sulphydryl alkylating agent that selectively uncouples pertussis toxin-sensitive G proteins from receptors (e.g. Shapiro *et al*., 1994; Isaacson, 1998). NEM attenuated opioid inhibition of I_{Ba} in our study, consistent with G-protein involvement in opioid inhibition. However, a direct effect of NEM on calcium channel gating, perhaps causing the slow baseline inhibition we observe (Figure 3e), cannot be ruled out (Yamaoka *et al*., 2000). Modification of responses by GTP-γ-S, as we observed, is also diagnostic for the involvement of G-proteins (Dolphin, 1998).

Comparison between striatum and NAc

The striatum and NAc have often been grouped under the term neostriatum because of anatomical and neurochemical similarities between neurons in the two regions (Hussain *et al*., 1996; Kawaguchi, 1997; Meredith *et al*., 1989; Wilson, 1998). Nevertheless, there is an increasing body of evidence for functional differences between the two regions. The striatum is well known to be involved in body movement and stereotyped behaviours and is more richly connected to motor areas of the CNS (e.g. Groves, 1983). In contrast, the NAc, especially the shell region, has been demonstrated to play a critical role in reward and addiction to substances of abuse. At the cellular level, there are differences in the actions of dopamine and noradrenaline on synaptic transmission (Nicola & Malenka, 1998) and of opioids on presynaptic inhibition and membrane potential (Jiang & North, 1992; Yuan *et al*., 1992).

Given these differences, it is interesting to note that our findings on the inhibition of calcium channels in the NAc are similar to those reported for some classes of neurons in the striatum. Opioids have been reported to inhibit calcium channels in striatal spiny neurons (Stefani *et al*., 1994). More recently, adenosine A_1 agonists have been shown to modulate calcium channels in cholinergic interneurons in the striatum (Song *et al*., 2000). Neuromodulatory actions of adenosine and opioids may therefore be relatively common in the neostriatum.

Mechanisms of withdrawal

Adenosine plays an important role in opioid-induced sensitization and opioid withdrawal (Dionyssopoulos *et al*., 1992; Kaplan & Sears, 1996; Salem & Hope, 1997; Weisberg & Kaplan, 1999). A robust upregulation of the cyclic-AMP cascade in the mesolimbic system of the NAc and VTA, following chronic administration of opioids and psychostimulants, has been well documented (Self & Nestler, 1995). These chronic drug treatments produce an elevated level of adenosine, thought to arise from the metabolism of cyclic-AMP (Bonci & Williams, 1996; Chieng & Williams, 1998). Adenosine appears to suppress withdrawal symptoms, like opioids (Dionyssopoulos *et al*., 1992; Kaplan & Sears, 1996; Salem & Hope, 1997). Our finding that opioids and adenosine have similar inhibitory effects on calcium channels in the same population of neurons suggests a possible cellular mechanism for the suppression of withdrawal. Further work needs to be done to examine this hypothesis.

Conclusions

The aim of this study was to characterize the effects of opioid- and adenosine-receptor agonists on calcium channels in neurons acutely dissociated from the shell region of NAc. We concluded that these agonists inhibit calcium channels in

presumed interneurons, with likely ramifications for the signalling properties of these cells. Since each interneuron is thought to regulate the excitability of a large number of projection neurons, this modulatory action of opioids and adenosine may be important for mediating opioid reward, sensitization, craving and withdrawal in the NAc. By understanding the mechanisms underlying these phenomena, new strategies for dealing with chronic opioid exposure may be developed, improving the management of drug abuse.

We thank Drs M. Connor and P. Osborne for their helpful comments. This work was supported by a C.J. Martin Fellowship to B. Chieng from the National Health and Medical Research Council of Australia, and by recurrent funding from the John Curtin School of Medical Research to J.M. Bekkers.

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(Received August 4, 2000
Revised February 27, 2001
Accepted March 9, 2001)