



GABA_B, opioid and α_2 receptor inhibition of calcium channels in acutely-dissociated locus coeruleus neurones

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1 The effects of GABA_B, opioid and α_2 receptor activation on different subtypes of calcium channels in acutely-dissociated rat locus coeruleus (LC) neurones were investigated using whole-cell patch clamping.

2 Barium currents through calcium channels could be fractionated into four classes: L-type (nimodipine-sensitive), N-type (ω -conotoxin GVIA-sensitive), P/Q-type (ω -agatoxin IVA-sensitive) and R-type (remaining in the presence of all three blockers). The percentage of each was, respectively, 25 ± 2 , 34 ± 1 , 28 ± 3 and $12 \pm 1\%$ (mean \pm s.e.mean, $n = 4$).

3 The GABA_B receptor agonist, baclofen, and the opioid receptor agonist, enkephalin, partially inhibited the total barium current in a concentration-dependent manner with EC₅₀ values of 2 and 0.3 μ M, respectively. Maximal inhibition was $17 \pm 1\%$ ($n = 38$) for baclofen and $30 \pm 2\%$ ($n = 20$) for enkephalin. The α_2 -adrenoceptor agonist, UK14304 (10 μ M), also inhibited barium current in these neurones ($28 \pm 2\%$, $n = 11$). The agonists did not shift the current-voltage relationship along the voltage axis.

4 Maximal baclofen inhibition of different calcium channel subtypes was $9 \pm 7\%$ (L-type, $n = 4$), $11 \pm 8\%$ (N-type, $n = 4$), $26 \pm 6\%$ (P/Q-type, $n = 4$), and $6 \pm 5\%$ (R-type, $n = 5$). The corresponding values for enkephalin inhibition were $5 \pm 9\%$ (L-type), $30 \pm 11\%$ (N-type), $37 \pm 9\%$ (P/Q-type), and $17 \pm 8\%$ (R-type).

5 In the presence of a saturating concentration of enkephalin, baclofen produced additional inhibition of the barium current. In contrast, in the presence of a saturating concentration of enkephalin, UK14304 produced no further inhibition of the barium current.

6 These results indicate that neuromodulation of calcium channels in LC neurones involves a complex pattern of overlapping and distinct second messenger pathways. Regulation of LC neuronal firing activity by the modulation of calcium channels may be important for LC-mediated behaviour such as alertness and vigilance.

Keywords: α_2 -Adrenoceptor; acutely dissociated; baclofen; calcium channel subtypes; calcium current; enkephalin; GABA_B receptor; locus coeruleus; opioid receptor

Abbreviations: α_2 receptor, alpha-2 adrenoceptor; Aga, ω -agatoxin IVA; Bac, baclofen; CTx, ω -conotoxin GVIA; Enk, methionine-enkephalin; F_L, fraction of calcium current in LC neurones mediated by L-type channels; GABA_B receptor, type B γ -aminobutyric acid receptor; I_L, per cent inhibition by baclofen of L-type calcium current; LC, locus coeruleus; L-type, L subtype of calcium channel; Nimo, nimodipine; N-type, N subtype of calcium channel; P/Q-type, P and Q subtypes of calcium channels; R-type, R subtype of calcium channel; UK, UK14304

Introduction

The locus coeruleus (LC) in the brainstem provides the most extensive noradrenergic projections in the central nervous system, with heavy contributions to the dorsal bundle and descending noradrenergic pathways. Factors that modulate the excitability of LC neurones will thus have important ramifications for the levels of noradrenaline in other parts of the brain. LC receives major excitatory afferents (glutamatergic) from nucleus paragigantocellularis and inhibitory afferents (GABAergic) from nucleus prepositus hypoglossi (Aston-Jones *et al.*, 1991b for review). Many of the LC afferents originating from these two medullary nuclei also contain the opioid receptor agonist, enkephalin (Aston-Jones *et al.*, 1991b; Van Bockstaele & Chan, 1997), suggesting the possibility of co-release of these neurotransmitters. Extracellular noradrenaline is also found in LC (Surprenant & Williams, 1987) where it is known to modulate potassium channels (North & Williams, 1985). Thus, at least GABA, enkephalin and noradrenaline are present in LC and so are

likely to be important for the physiological regulation of the excitability of LC neurones.

This idea has been tested by examining the effects of a number of neurotransmitters on the spontaneous firing of action potentials in LC neurones. Activation of GABA_B, μ -opioid, α_2 -adrenoceptor, somostatin, opioid-like orphan, and galanin receptors all inhibited firing by increasing an inwardly rectifying potassium conductance (Aghajanian & Wang, 1987; Connor *et al.*, 1996; Inoue *et al.*, 1988; North & Williams, 1985; Osmanovic & Shefner, 1988; Pieribone *et al.*, 1995). Stimulation of most of the above postsynaptic receptors has been demonstrated to open the same population of potassium channels (Aghajanian & Wang, 1987; Connor *et al.*, 1996; North & Williams, 1985), pointing to a convergence of the actions of different types of receptors.

We wished to ask whether another class of important ion channels, calcium channels, could also be modulated by the converging actions of the postsynaptic receptors on LC neurones. Calcium channels, like potassium channels, are involved in regulating neuronal excitability. There are a

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number of different subtypes of calcium channels, commonly distinguished by their sensitivity to selective blockers. Experiments done in the hippocampus and amygdala indicate that these calcium channel subtypes may be differentially modulated by a variety of neurotransmitters (Huang *et al.*, 1998; Scholz & Miller, 1996).

Here we explore the actions of GABA_B, opioid and α_2 -receptor stimulation on different subtypes of calcium channels in acutely-dissociated LC neurones. We show that GABA_B and opioid receptor activation inhibits some of the calcium channel subtypes to varying extents. Furthermore, opioid and α_2 receptor agonists inhibit calcium channels *via* a common pathway while opioid and GABA_B receptor agonists largely act *via* independent pathways.

Methods

Rat pups (3–9 days old) were used to make brainstem slices (300 μ m) containing the bilateral nuclei of LC. All procedures were approved by the Animal Experimentation Ethics Committee of the Australian National University. The brain was embedded in agar (1.5%) and then cut in chilled physiological saline using a vibratome. LC was micro-dissected from the slices under a stereo-microscope with a pair of fine hypodermic needles. Tissue containing LC was incubated in enzyme solution containing papain (20 units ml⁻¹) for 20 min at room temperature. The tissue was then triturated to dissociate individual neurones 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 Earle's Balanced Salt solution supplemented with 1 mM kynurenic acid to block glutamate receptors. LC neurones were identified with an inverted microscope and whole-cell recordings were made using a patch-clamp amplifier (Axopatch 1D). Patch pipettes of resistance 3–5 M Ω were filled with intracellular solution containing (in mM): CsCl 110, CaCl₂ 2, EGTA 10 (ethylene glycol-bis[β -aminoethyl ether]-N,N,N',N'-tetraacetic acid), Mg-ATP 5, Na-GTP 0.2, HEPES 10 (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]), pH 7.3. Acceptable access resistance was <20 M Ω , periodically monitored with repetitive 10 mV steps (20 ms duration). Series resistance compensation of 80% was used for all experiments. Voltage families of barium currents through calcium channels were evoked every 30 s. Linear leak currents and capacity transients were subtracted using a P/4 protocol. Drugs were dissolved in external solution and applied to the cell using a series of flow pipes. The external solution comprised (in mM): tetraethylammonium chloride 140, BaCl₂ 2, CsCl 5, HEPES 10, glucose 10, 0.05% bovine serum albumin, pH 7.3. Data was acquired using pClamp 6 and analysed with Axograph 4.0 (Axon Instruments). Sample means were statistically compared using a paired two tailed *t*-test with a significance level of *P* = 0.05.

Baclofen, methionine-enkephalin, kynurenic acid and papain were from Sigma (St Louis, MO, U.S.A.); nimodipine and UK14304 were from Research Biochemicals (Natick, MA, U.S.A.); CGP56999 and CGP35348 were gifts from Novartis (Switzerland); ω -conotoxin GVIA was from Alomone Labs (Jerusalem, Israel); and ω -agatoxin IVA was a gift from Pfizer Research (Groton, CT, U.S.A.).

Results

Acutely-dissociated LC neurones were identified as relatively large cells (soma diameter \sim 35 μ m) with three to six processes

(Ingram *et al.*, 1997). In current clamp, the cells fired action potentials spontaneously, as has been reported in both intact animals and *in vitro* brain slices (e.g. Aghajanian, 1978; North & Williams, 1985; Pepper & Henderson, 1980).

Different calcium channel subtypes are present in LC neurones

Barium currents flowing through calcium channels were evoked by stepping from a prepulse to -90 mV to a range of test potentials (-60 to $+60$ mV; Figure 1a and b). The maximal barium currents used in this study ranged from 0.5 to

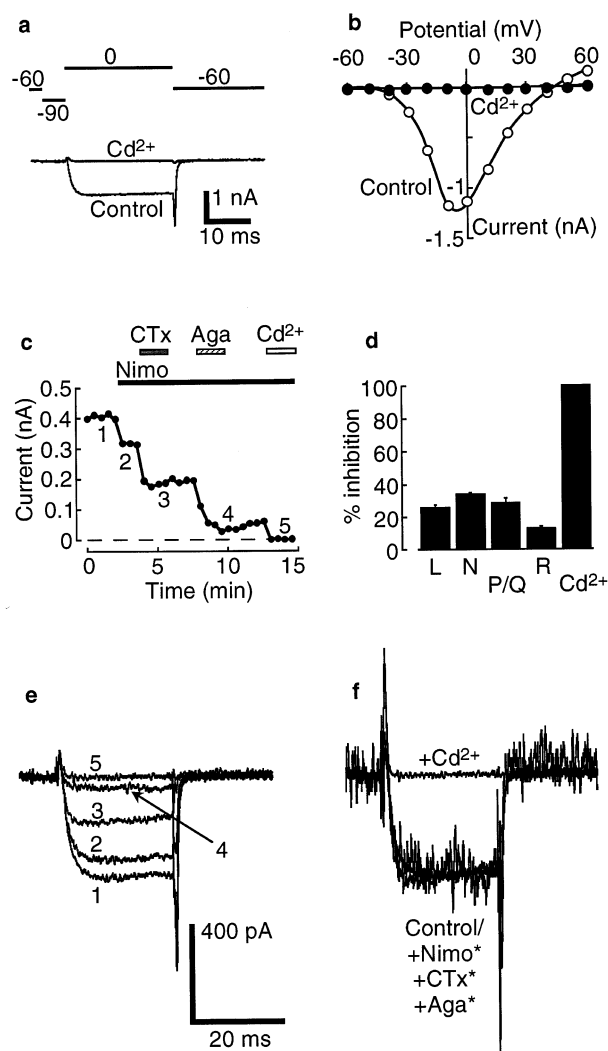


Figure 1 Four different subtypes of calcium channels can be distinguished in acutely-dissociated LC neurones. (a) Example of a barium current recorded during a test pulse to 0 mV from a prepulse of -90 mV (Control). This current was completely blocked by 100 μ M cadmium (Cd²⁺). The pulse protocol is 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 peak amplitude of barium current at 0 mV versus time during the experiment. Horizontal bars indicate periods of perfusion with nimodipine (Nimo, 3 μ M), ω -conotoxin GVIA (CTx, 1 μ M), ω -agatoxin IVA (Aga, 500 nM) and Cd²⁺ (100 μ M). Numbers 1–5 identify the example traces shown in (e). (d) Summary of the results of a series of four experiments as in (c), showing the fraction of current mediated by each of L-, N-, P/Q- and R-types of calcium channels. Error bars are \pm s.e. mean. (e) Example currents at the indicated time points in the experiment shown in (c). (f) Same traces as in (e); asterisks indicate that the traces are normalized to show the similarity of time courses.

3 nA (leak current subtracted). The current was completely blocked by 100 μM Cd^{2+} (Figure 1a and b). The current-voltage relationship for barium currents was an inverted bell-shaped curve with a peak at -10 to 0 mV (Figure 1b). This is consistent with other studies of neuronal barium and calcium currents (e.g. Connor & Christie, 1998; Ingram *et al.*, 1997).

The types of calcium channels present in the LC neurones were investigated using the subtype selective blockers, nimodipine (Nimo; 3 μM) for L-type channels, ω -conotoxin GVIA (CTx; 1 μM) for N-type channels and ω -agatoxin IVA (Aga; 500 nM) for P/Q-type channels (Figure 1c). The percentage of each was $25 \pm 2\%$ (L-type), $34 \pm 1\%$ (N-type) and $28 \pm 3\%$ (P/Q-type), leaving a residual (R-type) current of $12 \pm 1\%$ (mean \pm s.e. mean, $n=4$, Figure 1d). Superfusion of Cd^{2+} (100 μM) abolished the R-type current. Thus there was an approximately equal contribution of L-, N- and P/Q-types of calcium channels to the barium currents in these neurones.

Addition of the selective blockers did not alter the activation kinetics of the currents. All traces overlaid when they were scaled to the same amplitude (Figure 1e and f).

GABA_B, opioid and α_2 receptor agonists inhibited barium current

Baclofen reversibly reduced the amplitude of barium currents in a concentration-dependent manner (Figure 2) without shifting the current-voltage relationship along the voltage axis (Figure 2b). The activation kinetics were slowed in the presence of high concentrations of agonist (Figure 2a). This inhibition was abolished in the presence of GABA_B-receptor

antagonists, CGP56999 (100 nM) or CGP35348 (100 μM) ($n=5$), suggesting a GABA_B-receptor mediated action by baclofen (Figure 2c). The threshold for baclofen inhibition was about 100 nM and approached a maximum at 10 μM , with an EC_{50} of 2 μM (Figure 2e). This estimated EC_{50} value is the same as that for activation of potassium channels by baclofen in LC neurones (Osmanovic & Shefner, 1988). At a saturating concentration of baclofen (100 μM), barium current was inhibited by $17 \pm 1\%$ ($n=38$).

Like baclofen, the opioid receptor agonist, enkephalin, and the α_2 -adrenoceptor agonist, UK14304, reversibly inhibited barium current and slowed the activation kinetics, without shifting the current-voltage relationships (Figure 3). The mean inhibition of barium current by saturating concentrations of enkephalin (100 μM) and UK14304 (10 μM) were $30 \pm 2\%$ ($n=20$) and $28 \pm 2\%$ ($n=11$), respectively. From the enkephalin concentration-response curve, the EC_{50} was measured to be approximately 0.3 μM (*c.f.* ~ 0.5 μM for the effect of enkephalin on the potassium conductance in LC; North & Williams, 1985) (Figure 3d).

GABA_B and opioid receptor agonists inhibited different subtypes of calcium channels to different extents

We next asked whether baclofen- and enkephalin-activated receptors were coupled to different subtypes of calcium channels. R-type channels were isolated by superfusing a combination of Nimo (3 μM), CTx (1 μM) and Aga (500 nM). Baclofen (100 μM) inhibited the residual (R-type) current by $6 \pm 5\%$ ($n=5$) (Figure 4a, top right; Figure 4b). In the presence

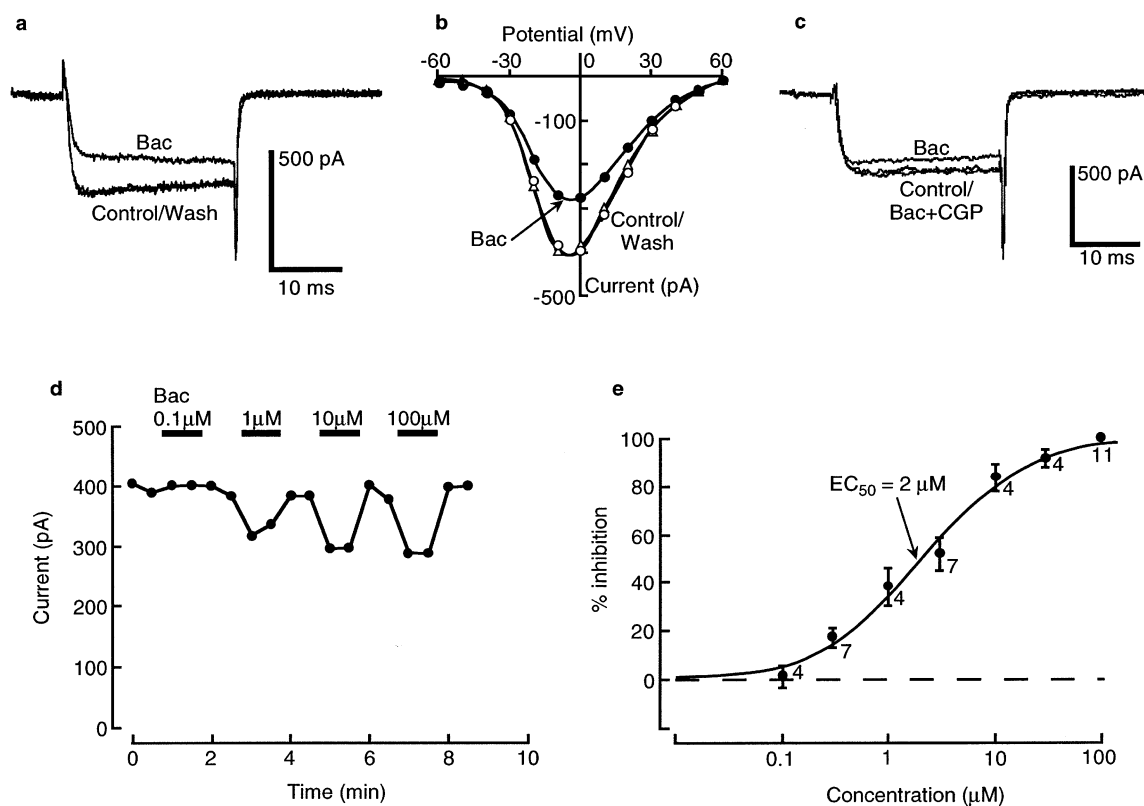


Figure 2 Baclofen (Bac) acts *via* GABA_B receptors to inhibit barium current in a concentration-dependent manner. (a) A typical barium current measured in control solution and after adding a saturating concentration of baclofen (100 μM). (b) Current-voltage (I-V) plot for the same cell as in (a), showing that baclofen does not cause a shift of the I-V plot along the voltage axis. (c) The inhibitory effect of baclofen (10 μM) is blocked by addition of a GABA_B receptor antagonist, CGP56999 (CGP, 100 nM). (d) Time course plot for peak barium current at 0 mV, in the indicated concentrations of baclofen. (e) Summary concentration-response for a series of experiments as in (d). The ordinate is expressed as percentage of maximal inhibition. The smooth curve is a fit of a logistic function, giving an EC_{50} of 2 μM . The numbers indicate n .

of CTx and Aga (L- plus R-type currents remaining), 100 μ M baclofen inhibited by $15 \pm 5\%$ ($n=4$). Thus the inhibition by baclofen of L-type current alone, obtained by subtracting the R-type inhibition, was estimated at $9 \pm 7\%$. Similar experiments were done to isolate the effect of 100 μ M baclofen on N- and P/Q-type currents by using the appropriate combinations of blockers. The measured inhibition was $17 \pm 7\%$ in Nimo plus Aga (N- plus R-types remaining; $n=4$) and $32 \pm 3\%$ in Nimo plus CTx (P/Q- plus R-types remaining; $n=4$). After subtracting the measured inhibition of pure R-type current, inhibition of N-type alone was estimated at $11 \pm 8\%$ and of P/Q-type alone at $26 \pm 6\%$.

The same series of experiments was done with 100 μ M enkephalin. The measured inhibition was $17 \pm 8\%$ (R; $n=5$), $21 \pm 5\%$ (L plus R; $n=4$), $46 \pm 9\%$ (N plus R; $n=4$) and $53 \pm 4\%$ (P/Q plus R; $n=4$). Following subtraction of the inhibition of pure R-type current ($17 \pm 8\%$), the estimated inhibition of each subtype alone was $5 \pm 9\%$ (L), $30 \pm 11\%$ (N), and $37 \pm 9\%$ (P/Q). All of the subtracted results are summarized in Figure 4b. Baclofen inhibits P/Q-type channels significantly more than R-type channels ($P<0.05$), and enkephalin inhibits P/Q-type channels more than L-type

channels ($P<0.05$). No other differences were found to be significant.

Effects of activation of opioid and α_2 receptors, but not opioid and GABA_B receptors, occluded one another

We next examined whether different receptor agonists had occlusive or additive effects on calcium channel inhibition. In the presence of baclofen (100 μ M), enkephalin (100 μ M) significantly inhibited the remaining barium current by a further 16%, to a total of $33 \pm 4\%$ for both drugs ($n=4$, Figure 5a and c). In the same cells, in the presence of enkephalin, baclofen consistently inhibited the remaining current by a further 10%, giving a total of $34 \pm 1\%$ for both drugs. In contrast, co-application of enkephalin (100 μ M) and UK14304 (10 μ M) produced little further inhibition beyond that

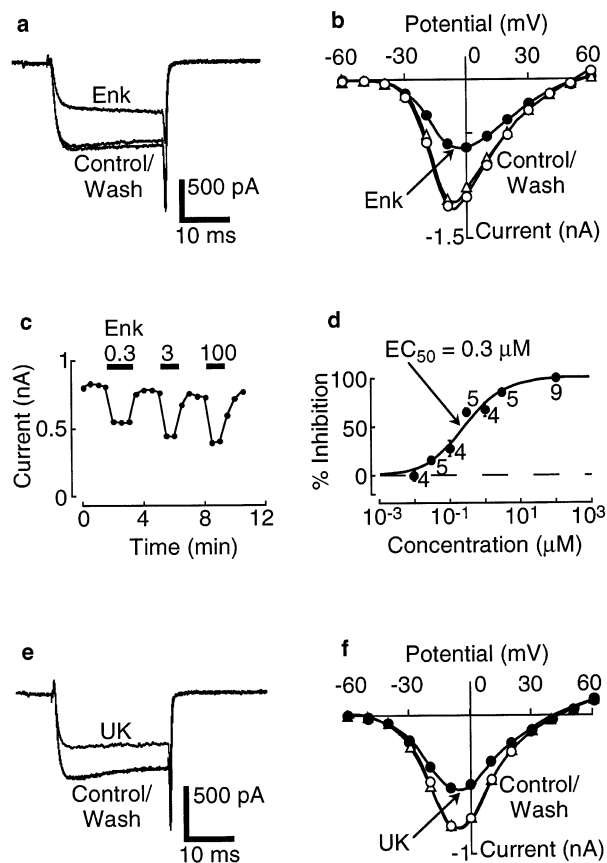


Figure 3 Enkephalin (Enk) acts *via* opioid receptors to inhibit barium current in a concentration-dependent manner. (a) Barium current measured in control solution and after adding a saturating concentration of enkephalin (100 μ M). (b) Current-voltage plot for the same cell as in (a), showing the lack of shift along the voltage axis following addition of enkephalin. (c) Time course plot for peak barium current at 0 mV, in the indicated concentrations of enkephalin. (d) Summary concentration-response plot for a series of experiments as in (c). The ordinate is expressed as percentage of maximal inhibition. The smooth curve is a fit of a logistic function, giving an EC_{50} of 0.3 μ M. The numbers indicate n . (e) Barium current measured in control solution and after adding a saturating concentration of UK14304 (UK, 10 μ M). (f) Current-voltage plot for the same cell as in (e).

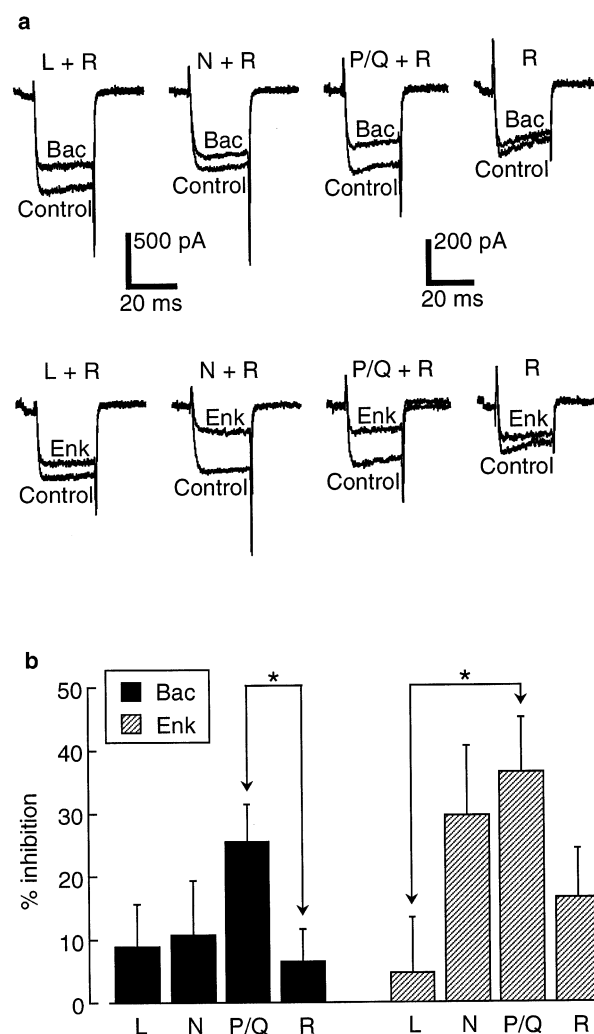


Figure 4 Baclofen (Bac) and enkephalin (Enk) inhibited different calcium channel subtypes to varying extents. (a) Example traces for the inhibitory effect of baclofen (100 μ M, top panels) or enkephalin (100 μ M, bottom panels) on the pharmacologically isolated subtypes of calcium channels. (b) Summary of the results of experiments as in (a). Inhibition of pure L-type currents was estimated by subtracting the measured inhibition of R-type currents (Nimo, CTx and Aga present) from that measured for L-type plus R-type currents (CTx and Aga present). A similar method was used to estimate the inhibition of pure N- and P/Q-type currents. A disadvantage of this subtraction procedure is that the error bars (\pm s.e.mean) are increased. Statistically significant differences in inhibition are indicated by the asterisks ($P<0.05$). $n=5$ for R-type measurements, $n=4$ for all others.

produced by each drug alone in the same cells ($25 \pm 2\%$ for enkephalin alone; $29 \pm 3\%$ for enkephalin plus UK14304; $29 \pm 2\%$ for UK14304 alone; $32 \pm 2\%$ for UK14304 plus enkephalin; $n=4$; Figure 5b and d). This suggests that some receptor types (e.g. opioid and α_2) activate converging pathways while others (e.g. opioid and GABA_B) do not.

Discussion

In this paper we have shown that activation of GABA_B, opioid and α_2 receptors inhibits barium current flowing through calcium channels in acutely-dissociated LC neurones (Figures 2 and 3). This inhibition occurred without shifting the current-voltage relationship along the voltage axis. GABA_B and opioid receptor activation inhibited some of the calcium channel subtypes to differing extents (Figure 4). Finally, the inhibitory effects of activation of GABA_B and opioid receptors were additive, whilst those of opioid and α_2 receptors were mutually occlusive (Figure 5).

At least four different classes of high voltage-activated (HVA) calcium channels are present in these cells, L, N, P/Q

(about 30% of each) and R (10%) (Figure 1d). Low voltage-activated T-type calcium channels are not apparent in LC neurones; test pulses to -30 mV from a strongly hyperpolarizing prepulse did not reveal a transient conductance (not illustrated). The activation kinetics of the different classes of HVA calcium currents were similar, since their normalized time courses were identical (Figure 1f). The duration of our test pulses was too short to allow comparison of inactivation time courses, which have been reported to differ between calcium channel subtypes (Catterall, 1995).

Receptor-mediated modulation of voltage-gated calcium channels has been intensively studied in a wide range of cell types (Hille, 1994; Zamponi & Snutch, 1998). The mechanism usually involves activation of intracellular G-proteins as second messengers. Although our experiments were not designed to directly address whether G-proteins are involved in the inhibition reported here, several features of the inhibition are typical of those involving G-proteins. The activation kinetics of barium currents were slowed following receptor activation (Figures 2a, 3a and e; Connor & Christie, 1998; Ingram *et al.*, 1997) and the inhibition could be removed by strong depolarization (Ingram *et al.*, 1997). Moreover, the pertussis toxin-sensitive G proteins, G_i and G_o, are known to be present in LC neurones, where they have been shown to mediate an inhibition of adenylate cyclase and an increase in inwardly rectifying potassium conductance (Christie, 1991).

A further characteristic of many G-protein mediated processes is the convergence of second messenger cascades, leading to occlusion between the effects of activation of different receptors (Simon *et al.*, 1991). Such occlusion was observed for the potassium conductance in LC neurones (Agahajanian & Wang, 1987; Connor *et al.*, 1996; North & Williams, 1985), and prompted us to look for similar effects on the calcium conductance in these cells. In one series of experiments, we studied the effects of GABA_B and opioid receptor activation on different calcium channel subtypes (Figure 4). The finding that these receptors inhibited some calcium channel subtypes to differing extents (Figure 4b) suggests a divergence in the actions of some second messenger cascades. This divergence is further highlighted by the finding that the inhibitory effects of activation of GABA_B and opioid receptors were additive (Figure 5a and c).

The results in Figure 5c can be predicted from those in Figures 1d and 4b if one assumes complete divergence of the actions of baclofen and enkephalin. Define F_L as the fraction of L-type calcium channels in LC neurones, obtained from Figure 1d. Similar terms can be defined for N-, P/Q- and R-types. Define I_L as the percentage inhibition of L-type calcium channels by baclofen, obtained from Figure 4b, and similarly for N-, P/Q- and R-types. The predicted baclofen inhibition of the total barium current is then $F_L I_L + F_N I_N + F_P I_P + F_R I_R = 14\%$, which is close to the experimental value of 17% (Figure 5c). A similar argument for enkephalin predicts inhibition of 24%, which is the same as the experimental value (Figure 5c). Assuming perfect independence of the actions of baclofen and enkephalin, the predicted co-inhibition by baclofen and enkephalin together is $14\% + 24\% = 38\%$ which is comparable with the observed value of 33% (Figure 5c). This analysis strongly indicates a lack of cross-talk between the actions of these two agonists. This contrasts with opioid and α_2 -receptor-mediated inhibition, which is mutually occlusive (Figure 5b and d), suggesting that activation of these receptors can lead to a convergence of second messenger cascades.

What mechanisms might explain divergence between the pathways activated by some combinations of receptor activation? Divergence could arise in one of three ways: (i)

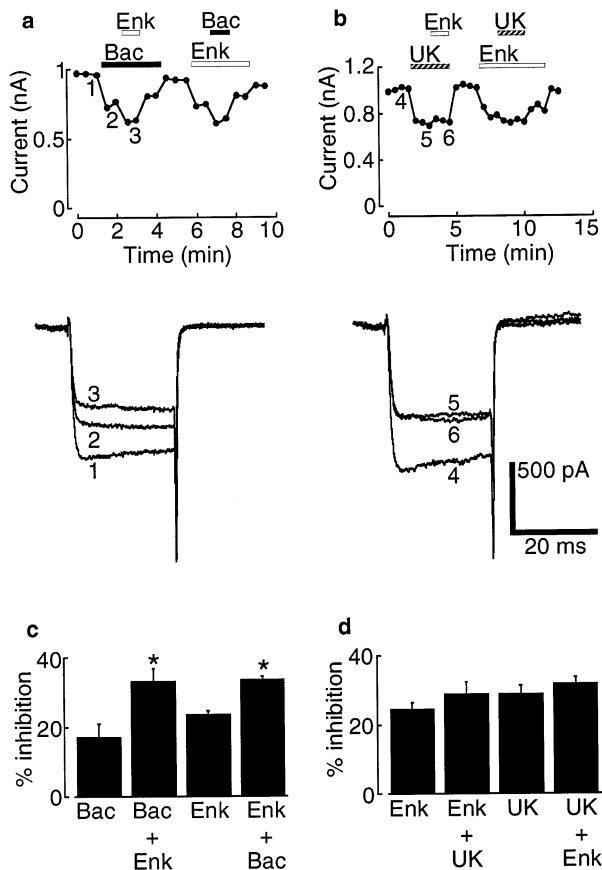


Figure 5 Saturating concentrations of baclofen (Bac) and enkephalin (Enk) produced additive inhibition of barium currents, whereas enkephalin and UK14304 (UK) produced mutually occlusive inhibition. (a and b) Upper panels. Time-course plots for peak barium currents at 0 mV showing the additive (left) and occlusive (right) effects of the indicated agonists. Lower panels. Example currents at the indicated time points on the time course plots. (c and d) Summary of the results of four experiments as in (a and b). The effects of baclofen and enkephalin were partially additive, whereas those of enkephalin and UK14304 were fully occlusive. Asterisks represent a significant difference ($P < 0.05$) between baclofen alone versus baclofen plus enkephalin, and enkephalin alone versus enkephalin plus baclofen.

agonists activate distinct second messengers that differ in their concentration or effectiveness at coupling to calcium channels; (ii) agonists activate the same second messengers but these are kept separate and couple to distinct populations of calcium channels; or (iii) agonists activate some combination of these two kinds of mechanism. In order to distinguish between these possibilities, future work would need to examine the identities and properties of the second messenger pathways involved in agonist-activated inhibition of calcium channels in LC neurones.

An important physiological role for LC neurones is to release noradrenaline from their extensive projections. This appears to underlie the functional behaviours involving the LC, such as arousal, attentiveness and antinociception (Aston-Jones *et al.*, 1991a; Foote *et al.*, 1991; Jones, 1991). Here we have studied the modulation of calcium channels found on the somas and proximal dendrites of acutely-dissociated LC neurones. These channels will contribute to the excitability of LC neurones, both directly, by their participation in calcium action potentials, and indirectly, through their role in synaptic integration (Yuste & Tank, 1996). Our results indicate that an array of endogenous agonists, including GABA, enkephalin

and noradrenaline, can suppress the excitability of LC neurones through their actions on calcium channels. If it is assumed that calcium channels located on the presynaptic terminals of LC neurones function in a similar way to somatic channels, our results indicate that the above agonists will directly inhibit the release of noradrenaline from LC terminals.

In conclusion, we have shown that three classes of agonists that are endogenous to LC, GABA, enkephalin and noradrenaline, exert a powerful inhibitory effect on calcium channels in acutely-dissociated LC neurones. Different agonists inhibit different calcium channel subtypes to varying extents, and the actions of some of the agonists are mutually occlusive. These properties will help to determine the role of LC neurones in normal brain processing.

We thank Dr Pankaj Sah for discussions and comments on the manuscript. B. Chieng is the recipient of a C.J. Martin Fellowship of the National Health and Medical Research Council of Australia. The donations of the CGP compounds by Novartis and ω -agatoxin IVA by Pfizer Research are gratefully acknowledged.

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(Received March 29, 1999

Revised April 24, 1999

Accepted May 5, 1999)