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$GABA_B$ receptor transduction mechanisms, and cross-talk between protein kinases A and C, in GABAergic terminals synapsing onto neurons of the rat nucleus basalis of Meynert

Hisahiko Kubota *, Shutaro Katsurabayashi *, Andrew J. Moorhouse †, Nobuya Murakami *, Hitoshi Koga * and Norio Akaike *‡

** Cellular and System Physiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan, † Department of Physiology and Pharmacology, School of Medical Sciences, University of New South Wales, Sydney, 2052 NSW, Australia and ‡ Research Division for Physiology and Pharmacology, Kumamoto Health Science University, Kumamoto 861-5533, Japan*

The transduction mechanisms underlying presynaptic $GABA_B$ receptor-mediated inhibition of transmitter release have been characterized for a variety of synapses in the central nervous system (CNS). These studies have suggested a range of transduction mechanisms, including a role for second messengers such as protein kinases A (PKA) and C (PKC). In the present study, we have examined the intracellular signalling pathways underlying baclofen-induced inhibition of GABA release from terminals synapsing onto rat basalis of Meynert neurons using patch-clamp recordings. Baclofen, a selective $GABA_B$ receptor agonist, reversibly decreased both evoked and spontaneous, miniature, GABAergic inhibitory postsynaptic currents (eIPSCs and mIPSCs, respectively). Such baclofen actions were completely abolished by CGP55845A, a selective $GABA_B$ receptor antagonist, and by staurosporine, a non-selective PKA and PKC inhibitor. The mIPSC frequency was still decreased by baclofen even in the presence of 4 AP, a K^+ channel blocker, and Cd^{2+}, a voltage-dependent calcium channel blocker. Pharmacological activation or inhibition of PKC activity affected basal GABA release and mildly affected the response to baclofen. Inhibition of the cAMP/PKA cascade also affected basal GABA release and, in a subset of neurons, occluded the effects of baclofen, suggesting that the $GABA_B$ receptor-mediated inhibitory action on GABA release was mediated via decreases in PKA activity. In addition, PKA inhibition occluded the effects of PKC modulation on both basal GABA release and on the response to baclofen. Our results characterize the transduction pathway of baclofen at these nucleus basalis of Maynert (nBM) synapses and show, for the first time, some cross-talk between the cAMP/PKA and PKC pathways in mammalian presynaptic nerve terminals.

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Corresponding author N. Akaike: Cellular and System Physiology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Fukuoka 812-8582, Japan. Email: akaike@physiol2.med.kyushu-u.ac.jp

γ-Amino-butyric acid (GABA) is a fast inhibitory transmitter in the mammalian CNS and activates both ionotropic $GABA_A$ and $GABA_C$ receptors and metabotropic $GABA_B$ receptors. Activation of presynaptic $GABA_B$ receptors elicits a reduction of neurotransmitter release at many central synapses (Nicoll *et al.* 1990). Baclofen, a selective $GABA_B$ receptor agonist, reduces neurotransmitter release in cerebellar and cultured sensory neurons whilst simultaneously inhibiting post-synaptic voltage-dependent Ca^{2+} channels (VDCCs) (Dittman & Rogehr, 1996; Harayama *et al.* 1998). Baclofen also hyperpolarizes hippocampal and substantia nigra neurons through activation of GTP-binding proteins (G-proteins) coupled to inwardly rectifying K^+ channels (GIRKs) (Misgeld *et al.* 1984; Gahwiler & Brown, 1985; Andrade *et al.* 1986; Lacey *et al.* 1988; Sodickson & Bean, 1996). An increase in K^+ conductance may also contribute to the presynaptic action of baclofen in hippocampal neurons because K^+ channel blockers reduce the extent of $GABA_B$ receptor-mediated presynaptic inhibition (Misgeld *et al.* 1989; Thompson & Gahwiler, 1992). Furthermore, $GABA_B$ receptors are negatively coupled to adenylate cyclase (AC) throughout the CNS (Kamatchi & Ticku, 1990; Knight & Bowery, 1996) and have also been suggested to modulate protein kinase C (PKC) activity in hippocampal and cultured spinal neurons (Dutar & Nicoll, 1988; Taniyama *et al.* 1992). The investigation of presynaptic $GABA_B$ receptor-mediated inhibition is also made somewhat more complex by the fact that pre- and postsynaptic $GABA_B$ receptors show differences in their pharmacology and transduction mechanisms (Dutar & Nicoll, 1988; Pitler & Alger, 1994; Yamada *et al.* 1999).

The nucleus basalis of Meynert (nBM) is composed of large cholinergic neurons and is the major source of

cholinergic inputs to the cerebral cortex in mammals (Johnston *et al.* 1979; Wenk *et al.* 1980). The excitability of nBM neurons is modulated by a range of different neuronal inputs, including GABAergic, noradrenergic, serotonergic, dopaminergic and glutamatergic afferent fibres arising from the nucleus accumbens, locus coeruleus, dorsal raphe nucleus, ventral tegmental area and cortex, respectively (Johnston *et al.* 1979; Wenk *et al.* 1980). We previously reported that the frequency of spontaneous GABAergic miniature inhibitory postsynaptic currents (mIPSCs), recorded in acutely dissociated rat nBM, neurons in which some functional synaptic boutons remained adherent to the isolated nerves, are modulated by activation of presynaptic $GABA_B$ receptors (Akaike *et al.* 1992) although the underlying mechanisms were not examined in that study.

In the present study, we initially focused on elucidating what transduction mechanisms underlie the baclofen-induced inhibition of GABA release from terminals synapsing onto nBM neurons, paying particular attention to the role(s) of PKC and PKA. We have also investigated the roles of these kinases in controlling the basal release of GABA. We utilized both the rat brain slice preparation and the 'synaptic bouton' preparation (Rhee *et al.* 1999; Akaike & Moorhouse, 2003), in which presynaptic modulation of neurotransmission can be investigated devoid of complications arising from surrounding neurons and glia. Our results also reveal some cross-talk, or interaction, between the PKA and PKC pathways in the presynaptic terminals.

METHODS

Neuronal preparations

We used the mechanically dissociated nerve–synaptic bouton preparation as originally described by Rhee *et al.* (1999) and recently reviewed (Akaike & Moorhouse, 2003). Briefly, 10- to 14-day-old Wistar rats were decapitated under pentobarbital anaesthesia. The brain was quickly removed and transversely sliced at a thickness of 400 μm with a Vibratome tissue slicer (VT 1000S, Leica, Germany). Slices were then kept in the incubation medium (see below) saturated with 95 % O_2–5 % CO_2 at room temperature (22–25 °C) for at least 1 h before dissociation. For dissociation, a slice was transferred into a 35 mm culture dish (Primatia 3801, Becton Dickinson, NJ, USA) and the region of nucleus basalis of Meynert (nBM) was identified under a binocular microscope (SMZ-1, Nikon, Tokyo, Japan). A fire-polished glass pipette was placed lightly on the surface of the nBM and was vibrated horizontally at 40–50 Hz for about 2 min using a custom-made vibration device. The mechanically dissociated neurons retained some of their original morphological features such as short portions of their proximal dendrites. Oval or triangular, medium-sized cells (15–20 μm in diameter) were randomly selected for the electrophysiological recordings.

For the slice preparations, coronal brain slices (250 μm thick) containing the nBM, were made from 10- to 14-day-old Wistar rats (killed as above) with a Vibratome tissue slicer (Leica). Slices were subsequently kept in the incubation medium at room temperature (22–25 °C) for at least 1 h before commencing recordings.

Electrical measurements

For the recordings from dissociated neurons we used the nystatin perforated patch-clamp recording mode (Akaike & Harata, 1994) whilst for recordings from neurons in the slice preparation we used conventional whole-cell patch-clamp recordings. All recordings were obtained under voltage-clamp conditions, at a holding potential (V_H) of −60 mV for the dissociated neurons and of –70 mV for the slice preparations, using a patch-clamp amplifier (EPC-7; List Electronic, Darmstadt, Germany). Patch pipettes were made from borosilicate capillary glass (1.5 mm o.d., 0.9 mm i.d., G 1.5, Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PB-7, Narishige). The resistance between the patch pipettes filled with internal solution and the reference electrode was 5–7 MΩ. Dissociated neurons were visualized using a phase-contrast inverted microscope (SMZ-1; Nikon) while the nBM neurons in the slice were identified using a water-immersion lens attached to an upright microscope (Axioscope, Zeiss, Germany). Current and voltage were continuously monitored on an oscilloscope (MS-5100A, Iwatsu, Tokyo, Japan) and recorded on a pen recorder (Recti-Horiz 8K, Nippondentki San-ei, Tokyo, Japan) and on a computer after digitizing at 4 kHz. All experiments were performed at room temperature (22–25 °C).

Data analysis

Spontaneous synaptic events were detected and analysed using MiniAnalysis software (Synaptosoft, Decatur, GA, USA) and IGOR PRO software (Wavemetrics, Lake Oswego, OR, USA). Spontaneous events were automatically screened using an amplitude threshold of 5 pA and then were visually accepted or rejected based upon the rise time and decay time. In more complex waveforms, where one spontaneous synaptic event occurred before the previous event had decayed back to the baseline, the baseline for the second event was estimated by extrapolating the decay of the first peak. Recordings included for data analysis were collected during periods of stable series resistance. In addition, recordings with leak current more than −50 pA (at V_H) were also discarded. The averaged values of mIPSC frequency and amplitude during the control period were calculated, and the frequency and amplitude of all events during and after drug application were normalized to these control values. Cumulative probability plots were constructed for mIPSC amplitude and frequency and were compared, under different experimental conditions, using the Kolmogorov-Smirnov test ($P < 0.05$). Mean mIPSC frequency and amplitude, recorded under the different experimental conditions, were compared using Student's *t* test.

In the slice preparations, synaptic currents were evoked by applying short (100 μs) stimulus voltage pulses (at a frequency of 0.1 Hz) to the glass stimulating pipette (Φ: 10 μm), which was filled with bath solution and placed near to the nBM neurons from which recordings were made. Stimulation protocols and data acquisition were performed using the PULSE program in the HEKA software package (HEKA).

Solutions

The ionic composition of the slice incubation medium (artificial cerebrospinal fluid) used during recordings was (mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 24 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose equilibrated with 95 % O_2–5 % CO_2. The pH value was 7.4. The standard external solution used during recordings from dissociated neurons was (mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$,

10 glucose and 10 Hepes. The pH of this external solution was adjusted to 7.4 with tris (hydroxymethyl) aminomethane (Tris-OH).

All external solutions routinely contained 3 μM 6 cyano-7-nitroquinoxaline-2,3 dione (CNQX) and 10 μM DL-2-amino-5-phosphonovaleric acid (AP5) to block any glutamatergic currents. 300 nM tetrodotoxin (TTX) was further added to block voltage-dependent Na^+ channels when recording spontaneous miniature currents (mIPSCs).

The ionic composition of the internal (patch-pipette) solution for the nystatin perforated patch recording was (mM): 20 *N*-methyl-D-glucamine methanesulfonate, 20 caesium methanesulfonate, 5 $MgCl_2$, 100 CsCl and 10 Hepes with pH adjusted to 7.2 with Tris-OH. Nystatin was dissolved in acidified methanol at 10 mg ml^{-1} and this stock solution was diluted with the internal solution just before use at a final concentration of 100–200 μg ml^{-1}. The ionic composition of the patch-pipette solution for the conventional whole cell patch recording was (mM): 43 CsCl, 92 caesium methanesulfonate, 5 tetraethyl-ammonium chloride, 2 EGTA, 1 $MgCl_2$, 4 ATP-Mg and 10 Hepes. The pH was adjusted to 7.2 with Tris-OH.

Drugs

Drugs used in the present study were baclofen, AP5, bicuculline, CNQX, *N*-ethylmaleimide (NEM), 4-aminopyridine (4 AP), staurosporine, SQ22526, chelerythrine and phorbol 12-myristate 13-acetate (PMA) (all from Sigma, St Louis, MO, USA) and TTX (Wako Pure Chemicals, Tokyo, Japan). CNQX was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and used as a stock solution. CGP55845A was generously provided by Novartis-Pharma (Basel, Switzerland). Drugs were applied using a 'Y-tube system', which enables rapid solution exchange.

RESULTS

Presynaptic inhibition of GABA release by baclofen

We initially confirmed that activation of GABA$_B$ receptors inhibited GABA release using the slice preparation. Evoked inhibitory postsynaptic currents (eIPSCs) were recorded at a V_H of –70 mV in response to electrical stimulation. We have previously categorized these eIPSCs, recorded under the same conditions, as being mediated via activation of GABA$_A$ receptors based on their complete block by 10 μM bicuculline (Rhee *et al.* 1999). We also confirmed their bicuculline sensitivity in a small number of recordings (e.g. Fig. 1*Ca*). Baclofen (10 μM), a selective GABA$_B$ receptor agonist, reversibly and significantly decreased the amplitude of these GABAergic eIPSCs, to about 25 % of the control eIPSC amplitude (Fig. 1*A*), without causing any change in their decay time (Fig. 1*B*). The baclofen-induced eIPSC inhibition was completely abolished by CGP55845A (10 μM), a selective GABA$_B$ receptor antagonist, which, by itself, had no effect on the control eIPSC amplitude (Fig. 1*A*).

To investigate further whether baclofen's effect was due to a decrease in presynaptic release of GABA, the relative amplitude of eIPSCs evoked by paired pulse stimulation, a well recognized assay for presynaptic actions, was examined (e.g. Wilcox & Dichter, 1994). At inter-pulse intervals ranging from 20 to 500 ms, paired pulse depression (PPD) was always observed (data not shown). The amplitude of the second eIPSC was 0.8 ± 0.1 times the amplitude of the first eIPSC at an inter-pulse interval of 40 ms. In the presence of baclofen, this PPD was converted to paired-pulse facilitation with the amplitude of the second eIPSC being 2.5 ± 0.4 times the amplitude of the first eIPSC (n=10, Fig. 1*C*). The data indicate that the ultimate locus of the action of baclofen is to cause a reduction in the probability of GABA release.

One explanation for the above effects is that presynaptic GABA$_B$ 'auto-receptors' regulate GABA release from synapses on to nBM neurons. To further address this, we recorded GABAergic spontaneous miniature inhibitory postsynaptic currents (mIPSCs) using the synaptic bouton preparation (Rhee *et al.* 1999; Akaike & Moorhouse, 2003), in which functional synaptic terminals remain adherent to the isolated neurons. All the following results were obtained using this preparation, and at a V_H of –60 mV.

In all nBM neurons, 1 μM baclofen decreased the frequency of mIPSCs (to 33.5 ± 6.9 % of the control frequency, n=15) without causing any change in the current amplitude (Fig. 2*A*). The mIPSC frequency recovered completely back to the control value within 10–15 min of washing out baclofen. We have previously reported that these mIPSCs, recorded under the same conditions, are completely blocked by bicuculline (Rhee *et al.* 1999) and this was again confirmed in three neurons in the present recordings (data not shown). Therefore, these spontaneous synaptic events represent pure GABAergic IPSCs. The inhibitory effect of baclofen on the frequency of mIPSCs was completely blocked by the selective GABA$_B$ receptor antagonist CGP55845A (1 μM), even when a slightly higher baclofen concentration (3 μM) was used (Fig. 2*B*). In addition, CGB55845A itself did not affect the control mIPSC frequency, suggesting that there was no tonic auto-inhibition of GABA release via basal activation of GABA$_B$ receptors in this preparation.

The effects of both baclofen and CGP55845A on the postsynaptic response to exogenous application of GABA were also examined. Repetitive applications of GABA (10 μM) produced inward currents with reasonably constant amplitudes, indicating no major 'run down' of responses, for up to 2 h at least. Neither baclofen nor CGP55845A had any effect on the basal membrane currents or on the GABA-induced inward currents (data not shown), indicating that there were no postsynaptic GABA$_B$ receptors present in these nBM neurons and that the action of baclofen on mIPSC frequency was mediated via activation of presynaptic GABA$_B$ receptors. Furthermore, it seems very reasonable that these presynaptic GABA$_B$ receptors were also mediating the baclofen-induced inhibition of eIPSCs observed in the slice preparations (Fig. 1).

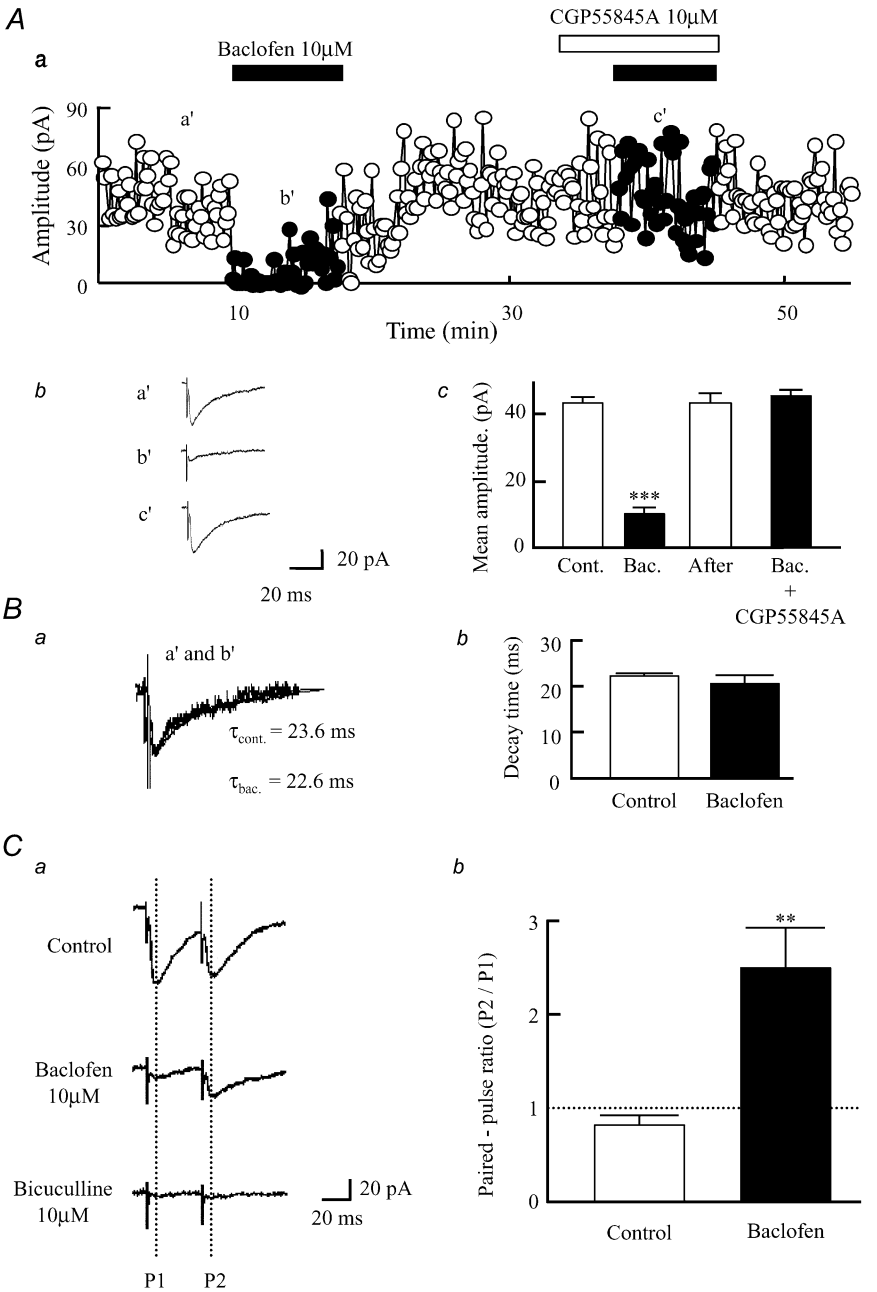

Figure 1. Effect of baclofen on stimulus-evoked IPSCs recorded in Meynert's neurons in rat brain slices

A, GABAergic evoked IPSCs (eIPSCs) recorded at a V_H of –70 mV before, during and after application of 10 μM baclofen and baclofen + 10 μM CGP55845A. A*a*, typical experiment showing the amplitude of GABAergic eIPSCs in different solutions as indicated. *b*, typical eIPSCs at times a′, b′ and c′ in *Aa*. *c*, mean amplitude of eIPSCs recorded during the control period (Cont.), in the presence of baclofen (Bac.), following washout of baclofen (After) and in the presence of baclofen and CGP55845A. Each column is the mean + S.E.M. of data from 4 neurons. (*** $P < 0.001$ *vs.*control, $n = 4$). *B*, effect of baclofen on eIPSC decay time. *a*, individual eIPSCs in control (a′) and in the presence of baclofen (b′). The amplitude of the eIPSC recorded in the presence of baclofen was scaled so as to equal the control eIPSC amplitude. *b*, mean decay time of eIPSCs with or without baclofen ($n = 4$). *C*, effects of baclofen on eIPSCs elicited by paired stimulation pulses. *a*, typical example of a pair of eIPSCs (P1 and P2) elicited at an inter-pulse interval of 40 ms in control (top), in the presence of baclofen (middle) and in the presence of bicuculline (bottom) ($n = 4$). *b*, mean amplitude histogram of the ratio of the second eIPSC relative to the first eIPSC, elicited at an inter-pulse interval of 40 ms with or without baclofen (** $P < 0.01$, $n = 4$).

Involvement of G-proteins in the action of baclofen

Previous reports have demonstrated that GABA$_B$ receptors in the CNS neurons are coupled to GTP-binding proteins (G-proteins) (Rohrbacher *et al.* 1997; Chen & Pol, 1998; Isaacson, 1998.). Hence, we examined whether G-proteins are also involved in the inhibitory effects of baclofen on GABA release onto nBM neurons. As shown in Fig. 3, *N*-ethylmaleimide (NEM, 10 μM), a selective uncoupler of pertussis toxin-sensitive G-proteins (G_i and G_o), increased the mIPSC frequency (to 202.5 ± 35.9 % of control, $P < 0.05$, $n = 4$) without affecting the distribution of current amplitudes. In the presence of NEM (10 μM), baclofen had no effect on mIPSC frequency or amplitude, even at a higher concentration (30 μM). The mIPSC frequency and amplitude in the presence of baclofen (30 μM) was, respectively, 99.8 ± 0.2 % of the mIPSC

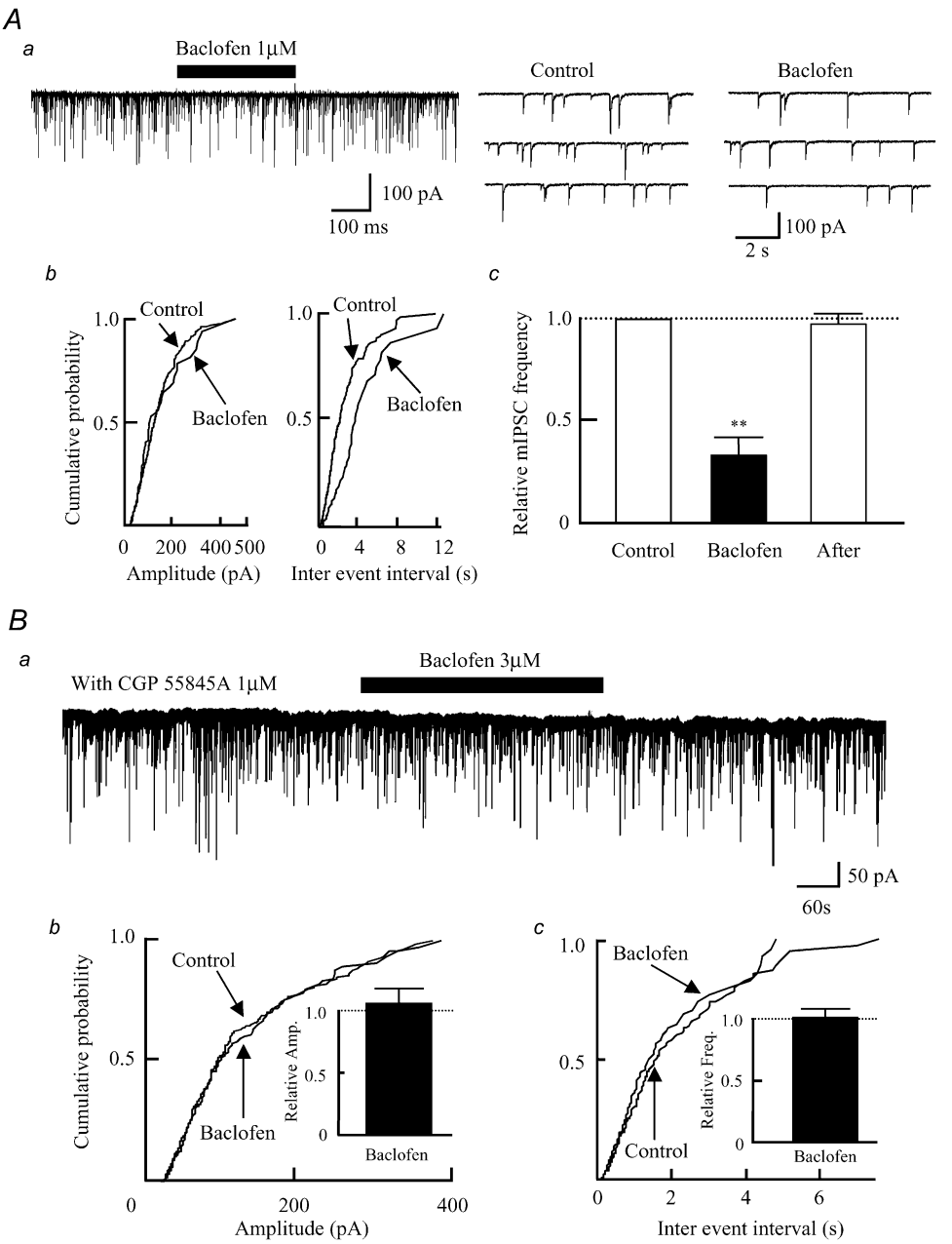

Figure 2. Inhibitory effect of baclofen on spontaneous mIPSCs

Aa, sample traces of mIPSCs before, during and after application of 1 μM baclofen. *b*, cumulative mIPSC amplitude (left) and frequency (right) distributions recorded in control and in the presence of baclofen. *c*, effect of baclofen on averaged mIPSC frequency (** $P < 0.01$, $n = 15$). *Ba*, typical trace of mIPSCs before, during and after the application of 3 μM baclofen in the presence of 1 μM CGP55845A. *b* and *c*, cumulative mIPSC amplitude (*b*) and frequency (*c*) distributions compiled from the trace indicated in Fig. 2*Ba*. Insets show the mean (+ S.E.M.) relative effect of baclofen ($n = 5$ neurons).

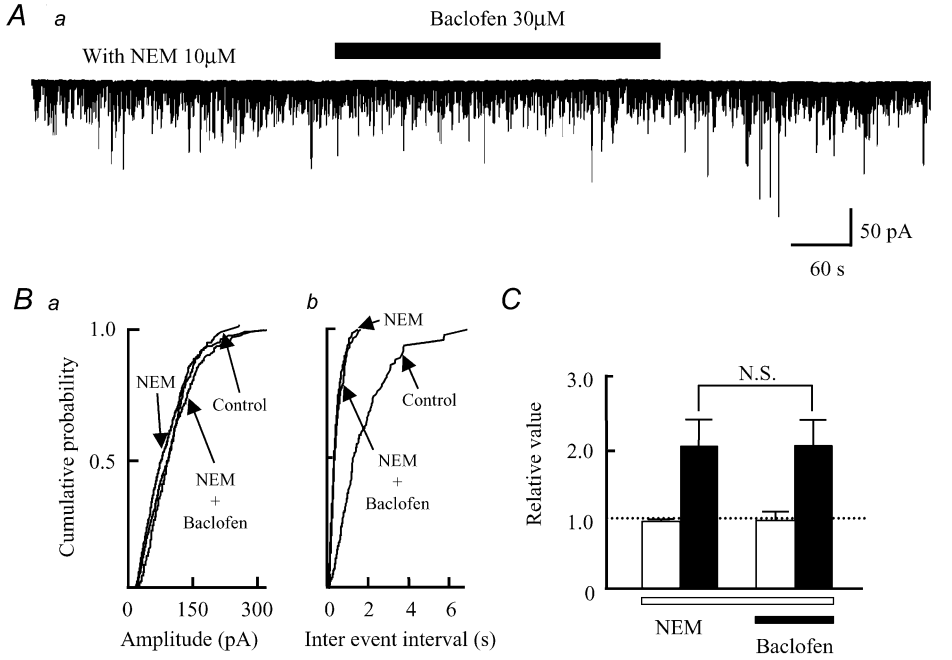

Figure 3. Blockade of baclofen action by NEM treatment

A, sample trace of mIPSCs recorded before, during and after the application of 30 μM baclofen in the presence of 10 μM NEM. *B*, typical cumulative mIPSC amplitude (*a*) and frequency (*b*) distributions with or without NEM and in the presence of both NEM and baclofen. *C*, mean mIPSC amplitude (□) and frequency (■), in control NEM and NEM + baclofen. Each column represents the mean + S.E.M. from 4 neurons. In this and subsequent figures, N.S. means no significant difference.

frequency in the presence of NEM, and 100.4 ± 10.0 % of the NEM control mIPSC amplitude (n = 4; Fig. 3*B*). These results clearly demonstrate that the inhibitory effect of baclofen on mIPSC frequency is mediated through pertussis toxin-sensitive G_i and G_o proteins.

Involvement of K^+ and Ca^{2+} channels in the action of baclofen

Activation of presynaptic $GABA_B$ receptors can cause activation of presynaptic K^+ channels and/or an inhibition of presynaptic Ca^{2+} channels, with both actions being mediated via G-proteins (Takahashi *et al.* 1998; Isaacson,

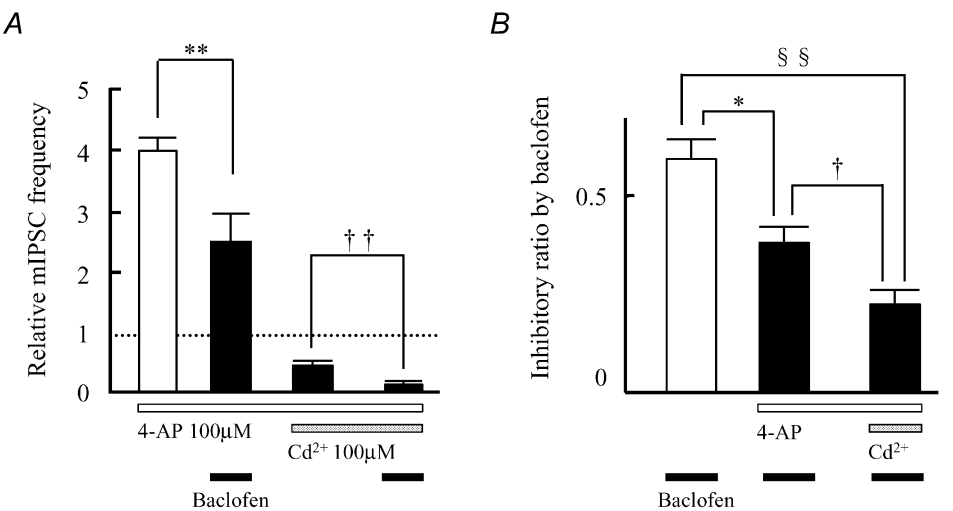

Figure 4. Baclofen action under K^+ and Ca^{2+} channel blockade

A, the effect of baclofen on mIPSC frequency in the presence of 100 μM 4 AP, 100 μM Cd^{2+}, or both. Each column represents the mean + S.E.M. of data from 4 neurons. ** $P < 0.01$ *vs.* 4 AP; †† $P < 0.01$ *vs.* 4 AP + Cd^{2+}. *B*, the inhibition ratio by baclofen. Each column is recalculated from *A*. * $P < 0.05$ *vs.* baclofen; † $P < 0.05$ *vs.* 4 AP + baclofen; §§ $P < 0.01$ *vs.* baclofen.

1998; Sun & Chiu, 1999). Thus, we examined whether these channels are involved in the inhibitory effects of baclofen on GABA release. 4 AP (100 μM), a K^+ channel blocker, increased the mIPSC frequency to 396.4 ± 13.8 % of the control value. In the presence of 4 AP, baclofen still reduced the mIPSC frequency (back to 253.3 ± 40.4 % of control, $P < 0.01$) as shown in Fig. 4A. In the presence of 4 AP, additional application of Cd^{2+} (100 μM), a non-selective voltage-dependent Ca^{2+} channel (VDCC) blocker, significantly decreased the mIPSC frequency, back to 51.8 ± 8.26 % of the original control frequency. In the presence of both 4 AP and Cd^{2+}, baclofen significantly further reduced mIPSC frequency (16.3 ± 1.44 %). In contrast, none of these experimental manipulations had any effect on the mean mIPSC amplitude (data not shown). Figure 4*B* shows the inhibition produced by baclofen, expressed relative to the frequency in the period immediately preceding baclofen application (the inhibitory ratio). When normalized in this way, baclofen reduced mIPSC frequency by 38.1 ± 8.7 and 22.5 ± 5.8 % in the presence of 4 AP and 4 AP + Cd^{2+}, respectively, which was significantly less than observed in the absence of these agents when baclofen reduced mIPSC frequency by 59.2 ± 4.43 %. These results show that baclofen can still significantly decrease GABA release when K^+ and Ca^{2+} channels are blocked. However, baclofen is less effective under these experimental conditions.

Involvement of protein kinase A and C in the action of baclofen

Pertussis toxin-sensitive G-proteins coupled to $GABA_B$ receptors (G_i) can inhibit adenylate cyclase and consequently reduce cAMP levels and protein kinase A (PKA) activity. This reduction in PKA activity has been reported to contribute to the inhibition of GABA release in response to $GABA_B$ receptor activation (Andrade *et al.* 1986; Kamatchi & Ticku, 1990). The protein kinase C

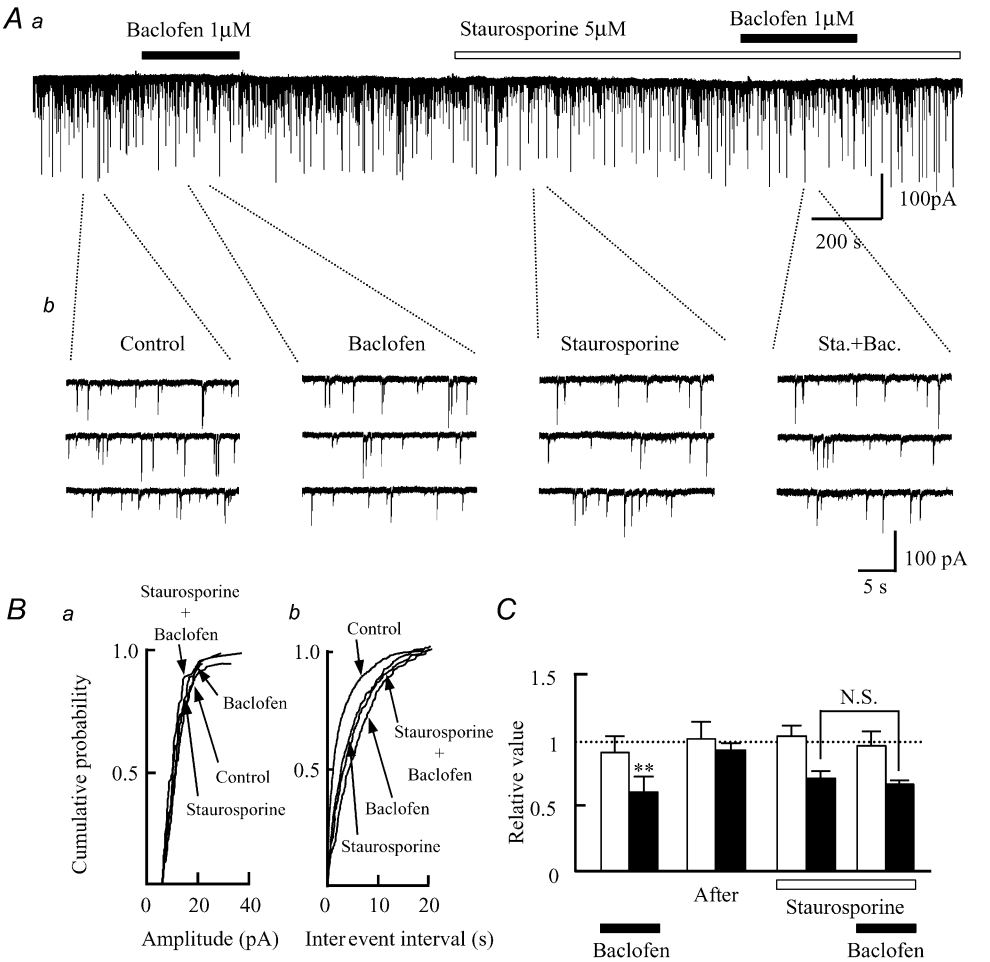

Figure 5. Blockade of baclofen action by staurosporine

Aa, sample trace of mIPSCs recorded in control conditions and in the presence of 1 μM baclofen, 5 μM staurosporine and baclofen + staurosporine (Sta. + Bac.). *b*, segments of the trace in *Aa* shown on an expanded time scale. *B*, cumulative mIPSC amplitude (*a*) and frequency (*b*) distributions recorded under the different experimental conditions. *C*, mean mIPSC amplitude (□) and frequency (■) in the presence of baclofen, following washout of baclofen and in the presence of staurosporine + baclofen. Each column represents the mean ± S.E.M. of 6 neurons, (** $P < 0.01$ *vs.* control).

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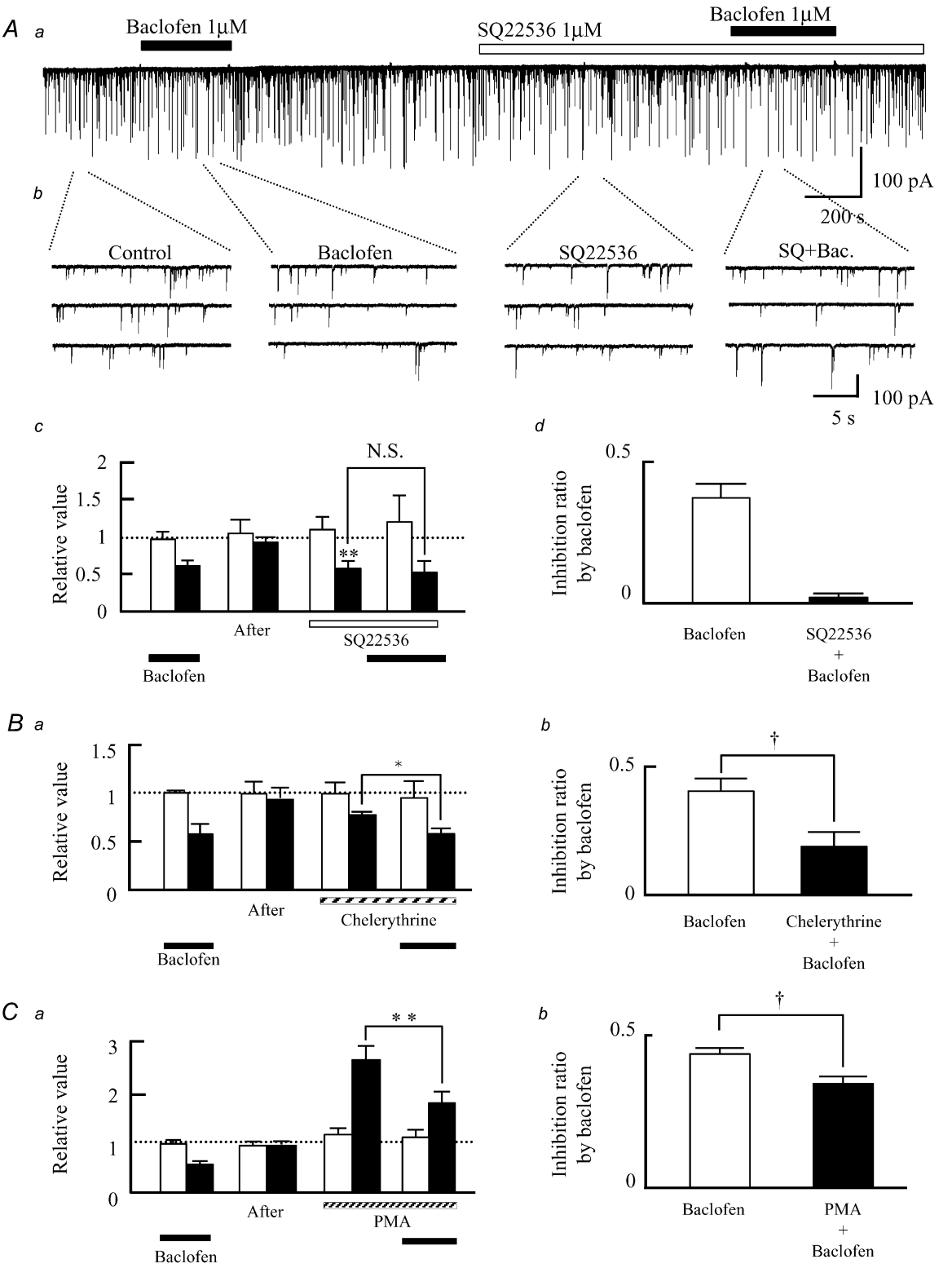

Figure 6. Effects of SQ22536, chelerythrine and PMA on mIPSCs

Aa, a typical trace of mIPSCs recorded in control, and in the presence of 1 μM baclofen, 1 μM SQ22536 and baclofen + SQ22536. *b*, segments of the trace in *Aa* shown using an expanded time scale. *c*, mean relative mIPSC amplitude and frequency in the presence of baclofen, following washout of baclofen and in the presence of SQ22536 and SQ22536 + baclofen. Each column represents the mean + S.E.M. of 5 neurons (** $P < 0.01$ *vs.* control). *d*, the inhibition ratio of baclofen, with or without SQ22536, on mIPSC frequency. Each column was recalculated from the data in *c*. *Ba*, mean mIPSC amplitude (□) and frequency (■) in the presence of baclofen, chelerythrine and chelerythrine + baclofen ($n = 6$, * $P < 0.05$ *vs.* control). *b*, the mIPSC

(PKC) cascade also participates in the inhibitory actions of baclofen within the hippocampus and in cultured spinal neurons (Dutar & Nicoll, 1988; Taniyama *et al.* 1992). To elucidate whether any or both of these kinases are involved in the inhibitory action of GABA$_B$ receptor activation on GABA release from synapses on to nBM neurons, we examined the effects of staurosporine, a non-selective PKA and PKC inhibitor (Fig. 5). By itself, staurosporine (5 μM) significantly reduced mIPSC frequency (to 71.0 ± 4.3 % of control, n=6, $P < 0.05$) without changing mIPSC amplitude, suggesting that either PKA or PKC, or both, regulate the basal, spontaneous release of GABA from these synapses (Fig. 5). In the presence of staurosporine, 1 μM baclofen had no affect on mIPSC frequency (which was 96.7 ± 0.25 % of that observed in staurosporine, n=6, Fig. 5*C*). These results suggest that the GABA$_B$ receptor-mediated inhibition of GABA release is mediated through either PKA or PKC, or through both.

Since adenylate cyclase (AC) produces intracellular cAMP from ATP, and cAMP activates PKA, the AC inhibitor SQ22536 would be expected to eventually completely block the PKA pathway. In 5 out of 11 neurons tested, SQ22536, by itself, decreased mIPSC frequency (to 57.0 ± 16.8 % of control, Fig. 6*A*). In this subset of cells, in the continued presence of SQ22536, 1 μM baclofen again had no effect on the mIPSC frequency (which was 98.4 ± 1.3 % of that in SQ22536 alone). In the remaining subset of six neurons, mIPSC frequency was actually increased in the presence of SQ22536 (data not shown). In this present report, we have not further characterized the underlying reasons for these different responses, or for the effects of baclofen in these cells (although it seems that baclofen was still effective in decreasing sIPSC frequency in these neurons).

The possible contribution of PKC to basal GABA release, and to the inhibitory effect of baclofen, was investigated by using a PKC inhibitor, chelerythrine, and a PKC activator, phorbol 12-myristate 13-acetate (PMA). Chelerythrine (1 μM) significantly decreased the basal mIPSC frequency, to 78.0 ± 2.1 % of the control value ($P < 0.01$, n=6, Fig. 6*B*). In the presence of chelerythrin, however, application of 1 μM baclofen still reduced the mIPSC frequency to 80.8 ± 5.5 % of that observed in the presence of chelerythrine alone (giving an inhibition of 19.2 ± 5.5 % compared to an inhibition of 44.2 ± 9.2 % in the original control conditions, Fig. 6*Bb*).

We further investigated the role of PKC (in nine other neurons) by first stimulating the kinase with PMA (1 μM). In the presence of PMA, mIPSC frequency was markedly increased to 262.6 ± 25.7 % of the control frequency (Fig. 6*C*). In the presence of PMA, however, baclofen (1 μM) still caused a significant decrease in mIPSC frequency, to 67.6 ± 15.1 % of the mIPSC frequency in PMA alone ($P < 0.01$, Fig. 6*Ca*). This represented an inhibition of 34.3 ± 3.41 % compared to an inhibition of 44.2 ± 2.46 % in the presence of PMA ($P < 0.05$, Fig. 6*Cb*). These results suggest that both the cAMP/PKA and PKC cascades affect the basal release of GABA and both contribute to the inhibition of release in response to baclofen. The contribution of the cAMP/PKA pathway to the effect of baclofen, however, markedly dominates over the contribution of PKC.

Blocking cAMP/PKA occludes the actions of PKC

As both the cAMP/PKA system and PKC seemed to contribute to basal GABA release, we next investigated whether these two systems acted independently, or whether some sort of 'cross-talk' existed between them. To investigate this, we manipulated one pathway and then the next, initially investigating PKC modulation when the cAMP/PKA pathway was blocked. In the presence of the adenylate cyclase blocker SQ22536 (1 μM), basal mIPSC frequency was again significantly decreased to 67.9 ± 3.8 % of the control frequency (Fig. 7*A*; observed in six out of nine neurons). Only those cells which responded to SQ22536 with an inhibition of GABA release were used for these experiments. The subsequent co-application of the PKC inhibitor chelerythrine no longer reduced GABA release, with mIPSC frequency being 98.2 ± 1.2 % of that observed in SQ22536 alone (n = 6; Fig. 7*Aa*). Likewise, in the continued presence of SQ22536 the application of PMA also no longer caused an increase in mIPSC frequency (mIPSC frequency with PMA was 106.2 ± 8.0 % of that in SQ22536 alone, Fig. 7*Ab*; n = 7).

We next changed the protocol, testing the effect of SQ22536 on GABA release when PKC was either inhibited or stimulated. The application of chelerythrine (1 μM) caused a decrease in mIPSC frequency to 65 ± 2.7 % of the control value ($P < 0.001$, n = 5; Fig. 7*Ba*), similar to that previously observed (Fig. 6). The subsequent co-application of SQ22536 (1 μM) further decreased mIPSC frequency in all six cells to 72.4 ± 4.8 % of that observed in the presence of chelerythrine alone ($P < 0.05$). As previously observed (Fig. 6*C*), PMA (1 μM) greatly facilitated mIPSC frequency to 215 ± 17.8 % of the control ($P < 0.001$; Fig. 7*Bb*). In the continued presence of PMA, co-application of SQ22536 (1 μM) could still decrease mIPSC frequency to 78.4 ± 6.5 % of that observed in PMA

inhibition ratio by baclofen with or without chelerythrine. Each column was recalculated from the data in *a* († $P < 0.05$ *vs.* baclofen). *Ca*, mean mIPSC amplitude and frequency in the presence of baclofen, PMA and PMA + baclofen (n = 9, ** $P < 0.01$ *vs.* PMA). *b*, the mIPSC inhibition ratio by baclofen with or without PMA. Each column was recalculated from the data in *a* († $P < 0.05$ *vs.* baclofen).

alone ($P < 0.05$; in all seven cells). The mean mIPSC amplitude was not significantly changed throughout these experiments. These results indicate that modification of the PKC cascade has no effect on GABA release when the cAMP/PKA system is blocked, suggesting that the actions of PKC may be mediated through the cAMP/PKA pathway. On the other hand, inhibiting or stimulating PKC does not seem to markedly change the effects of cAMP/PKA inhibition on GABA release.

We further investigated whether inhibition of the PKA system could occlude the effects of PKC manipulation on the baclofen response. When a subset of cells were first incubated with SQ22536, baclofen had no effect on mIPSC frequency (as shown previously, Fig. 6*A*). In these cells, in the continued presence of SQ22536, the co-application of either chelerythrine or PMA also no longer had any effect on the response to baclofen (Fig. 8*A* and *B*). Hence, in the presence of PKA inhibition, PKC modulation no longer affects the baclofen response, suggesting that PKC may also act via PKA to modulate $GABA_B$ receptor responses (as suggested for basal GABA release, Fig. 7*A*). Furthermore, when cells were incubated with the PKC inhibitor chelerythrine or with the activator PMA, the subsequent application of SQ22536 could still occlude the effect of baclofen on mIPSC frequency (Fig. 8*C* and *D*). In the presence of both chelerythrine and SQ22536, baclofen reduced mIPSC frequency to 95.8 ± 1.1 % ($n = 5$; Fig. 8*C*), whilst in the presence of PMA and SQ22536, baclofen reduced mIPSC frequency to 94.8 ± 3.0 % ($n = 7$; Fig. 8*D*). This further supports our conclusion that cAMP/PKA is the dominant transduction pathway mediating the effects of baclofen in these neurons and again shows that PKC manipulation has no effect on baclofen response when PKA is blocked.

DISCUSSION

In the present study we have investigated the transduction pathways involved in the presynaptic $GABA_B$ receptor-mediated inhibition of GABA release from terminals impinging onto rat nBM neurons. In addition, we report a robust form of 'cross-talk' between the cAMP/PKA and PKC transduction pathways in these presynaptic terminals.

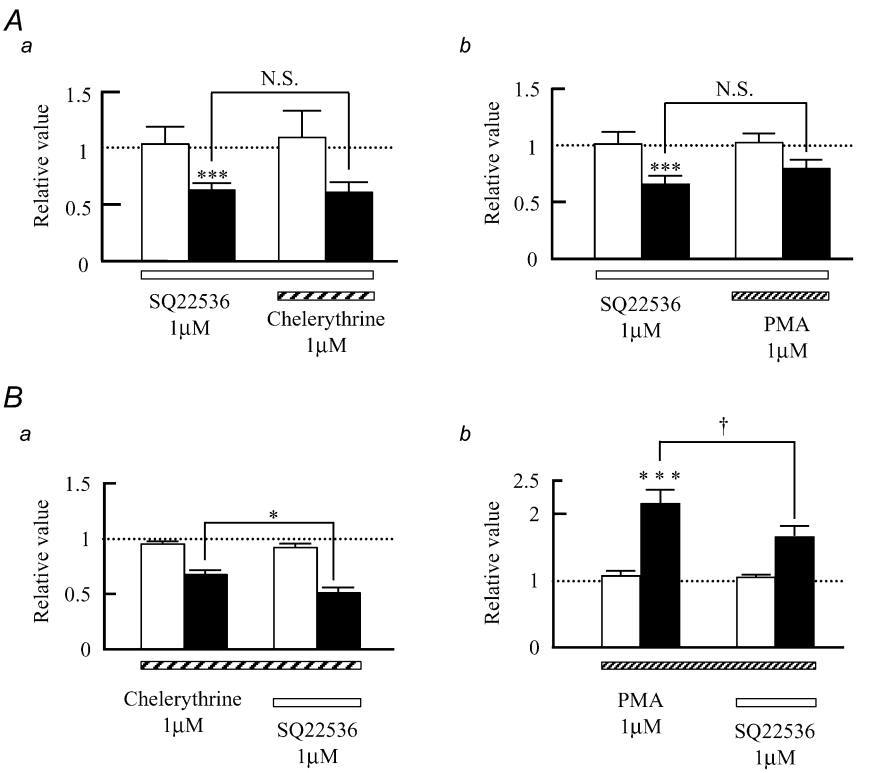

Figure 7. Inhibition of adenylate cyclase occludes the effects of manipulations of PKC activity

Aa, mean relative mIPSC frequency and amplitude in the presence of 1 μM SQ22536 and SQ22536 + 1 μM chelerythrine. Each column shows the mean ± S.E.M. of mIPSC amplitude and frequency, expressed relative to control values (*** $P < 0.001$ *vs.* control, $n = 6$). *b*, mean relative mIPSC frequency and amplitude in the presence of SQ22536 and SQ22536 + 1 μM PMA (***$P < 0.001$ *vs.* control, $n = 5$). *Ba*, mean relative effects on mIPSC frequency and amplitude of adenylate cyclase inhibition by SQ22536 in the presence of chelerythrine (* $P < 0.05$ *vs.* chelerythrine alone, $n = 6$). *b*, mean relative mIPSC frequency and amplitude in the presence of PMA and PMA + SQ22536 (*** $P < 0.001$ *vs.* control, † $P < 0.05$ *vs.* PMA alone $n = 7$). □, amplitude; ■, frequency.

Potential $GABA_B$ autoreceptor transduction pathways

Previous studies have demonstrated that postsynaptic $GABA_B$ receptors modulate neuronal excitability by activation of K^+ channels, inhibition of VDCCs and attenuation of kinase activities (Misgeld *et al.* 1989; Wu & Saggau, 1997). The G-protein-dependent transduction pathway linking receptor activation to these effectors is less clearly defined and direct. Membrane-bound coupling between G-proteins and channels, inhibition of the cAMP/PKA pathway and the PKC cascade have all been implicated as contributing to presynaptic $GABA_B$ receptor-induced decrease in neurotransmission at different synapses (Dutar & Nicoll, 1988; Taniyama *et al.* 1992; Takahashi *et al.* 1998).

Contribution of voltage-dependent K^+ and Ca^{2+} channels to the action of baclofen

VDCCs, which aggregate around the active zone in the presynaptic nerve terminal, are integral to neurotransmitter release (Doze *et al.* 1995; Takahashi *et al.* 1998). Activation of $GABA_B$ receptors has been demonstrated to inhibit Ca^{2+} influx through VDCCs in the nerve terminals, resulting in a decrease in neurotransmitter release (Dittman & Rogehr, 1996; Harayama *et al.* 1998). $GABA_B$ receptor activation has also been reported to hyperpolarize the postsynaptic membrane due to the direct effect of increasing K^+ conductance (Newberry & Nicoll, 1984; Gahwiler & Brown, 1985; Stevens *et al.* 1985; Osmanovic & Shefner, 1988). Increase of K^+ conductance by baclofen has also been reported to occur in some GABAergic presynaptic nerve terminals,

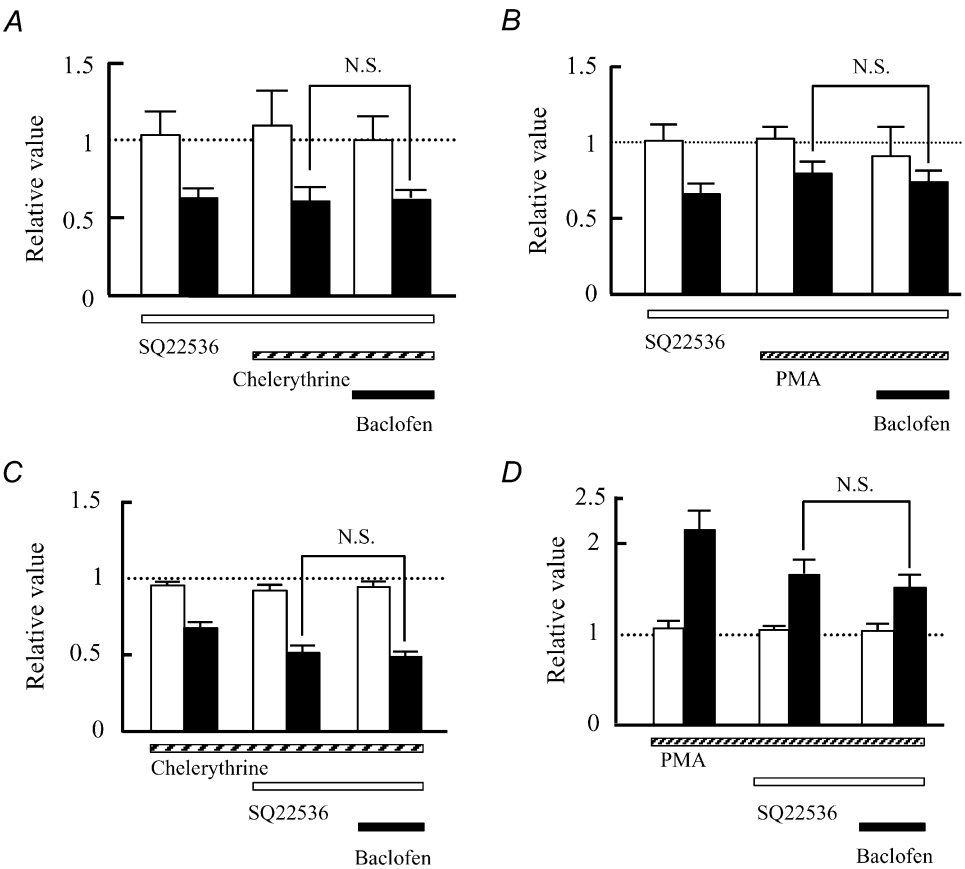

Figure 8. Effects of baclofen on mIPSCs recorded in the presence of various combinations of SQ22536, chelerythrine, and PMA

A, mean mIPSC amplitude and frequency in recordings in which SQ22536 caused a decrease in mIPSC frequency. The subsequent addition of 1 μM chelerythrine or chelerythrine + baclofen, in the continued presence of SQ22536, had no further effect on mISPCs. Each column represents the mean ± S.E.M. of data from 6 neurons. *B*, effects of PMA and PMA + baclofen on mean mIPSC amplitude and frequency in the continued presence of SQ22536. The subsequent addition of PMA, and then baclofen had no further effect on mIPSCs. Each column represents the mean + S.E.M. from 5 neurons. *C*, the effect of SQ22536 and subsequently baclofen on mean mIPSC frequency and amplitude in the continued presence of chelerythrine ($n = 6$). *D*, the effect of SQ22536 and subsequently baclofen on mean mIPSC frequency and amplitude in the continued presence of PMA ($n = 7$). □, amplitude; ■, frequency.

resulting in the reduction of the GABA release (Misgeld *et al.* 1989; Thompson & Gahwiler, 1992). These inhibitory effects are mediated via direct actions of the activated G-proteins. In the present study, the action of baclofen was still observed under blockade of both K^+ and Ca^{2+} channels. Furthermore, baclofen could still decrease the mIPSC frequency in Ca^{2+}-free external solution (data not shown). The inhibition ratio by baclofen was significantly decreased, however, by blockade of these ion channels, with baclofen being about half as effective as in the absence of these blockers. The results indicate that these channels contribute to the effect of baclofen but are not responsible for all of this. Because the inhibitory action of baclofen in the present study was totally abolished by kinase inhibition with staurosporine, it seems that the actions of baclofen on presynaptic K^+ and Ca^{2+} channels are mediated via phosphorylation by presynaptic kinases.

Contributions of PKA and PKC to the action of baclofen

When the cAMP/PKA cascade was blocked with SQ22536, the actions of baclofen were totally occluded, at least for the subset of recordings that are presented here. Hence the effects of baclofen at these synapses appear to be mediated via activation of G_i and an inhibition of adenylate cyclase and PKA activity. PKA activation is known to enhance currents through L-type Ca^{2+} channels (Catterral, 2000), which are responsible for sustaining about 40 % of mIPSC release at these synapses (Rhee *et al.* 1999). Our results suggest that inhibition of VDCC currents, however, contributed only partially to the effects of baclofen and suggest some more direct effect(s) of baclofen (via PKA inhibition) on GABA release. PKA activation is known to affect the release machinery to facilitate neurotransmission at various synapses, including the facilitation of mIPSC frequency (Capogna *et al.* 1995). One recently described potential target mediating these effects of PKA is the presynaptic protein synapsin, which is a snare-associated protein implicated in exocytosis and vesicle priming (Chheda *et al.* 2001).

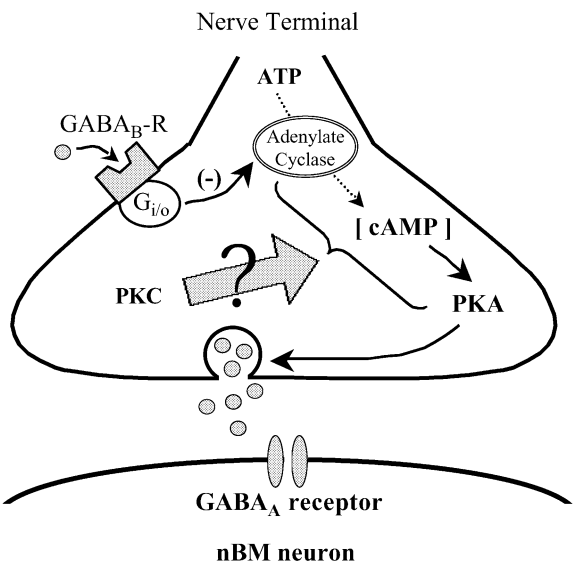

Figure 9. Schematic illustration of the main intracellular signal transduction pathways underlying the effects of presynaptic GABA$_B$ receptor activation

Presynaptic $GABA_B$ receptors couple with $G_{i/o}$ proteins to inhibit adenylate cyclase, which, via decreases in cAMP levels and PKA activity, reduces the GABA release. The PKC cascade is hypothesized to have no direct effects on GABA release, but instead affects GABA release (and the $GABA_B$ receptor transduction pathway) via cross-talk with the cAMP/PKA cascade through an unknown mechanism.

Our results also indicate that inhibition, or activation, of PKC reduced the inhibitory effects of baclofen. There are some reports that the $\beta\gamma$ subunits of G-proteins can activate phospholipase C and, hence, also activate PKC (Camps *et al.* 1992; Hamm, 1998). In addition, both pre- and postsynaptic $GABA_B$ receptor activation have been suggested to activate PKC in a number of CNS preparations (Kamatchi & Ticku, 1990; Jarolimek & Misgeld, 1997). Given that PKC activation with PMA enhanced GABA release, however, this particular transduction pathway does not seem to mediate these effects on the baclofen response. Furthermore, manipulation of PKC had no effect on the baclofen response when the cAMP/PKA pathway was blocked. This suggests that PKC is modulating the baclofen response via some cross-talk with the PKA pathway.

Cross-talk between PKA and PKC in presynaptic terminals

The activity of protein kinases within the presynaptic nerve terminals has important implications for transmitter release and for modulation of neurotransmission in the CNS (Greengard *et al.* 1993; Capogna *et al.* 1995). The release of GABA from synapses onto Meynert's neurons was also affected by kinase activity, being decreased in response to PKA inhibition (at least in the subset of neurons described here) and being decreased or increased, respectively, in response to PKC inhibition or activation. When the cAMP/PKA cascade was inhibited by SQ22536, however, manipulating the PKC pathway had no effect on GABA release. We conclude that PKC manipulation is acting on GABA release via the PKA pathway. This is the first demonstration of such cross-talk in mammalian presynaptic nerve terminals, although a somewhat similar observation has been reported in *Aplysia* sensory neurons using a different experimental strategy (Sugita *et al.* 1997). In that study, PKC activation caused an increase in cAMP levels and reduced the effects of PKA activation. Similar PKC-induced elevations in cAMP have been observed in a variety of non-neuronal cells, which have been attributed to PKC-induced increases in adenylate cyclase activity (e.g. Fukushima *et al.* 1996; Byung-Chang *et al.* 1998). Whilst

the exact nature of the cross-talk between PKA and PKC observed in the present study is unknown, our data are consistent with PKC mediating both its direct effects on GABA release, and its modulation of the baclofen response, also via an increase in cAMP levels.

Conclusion

Figure 9 shows a representation of a plausible scheme to explain our results. Activation of presynaptic $GABA_B$ receptors at some synapses inhibits adenylate cyclase, decreasing cAMP levels and PKA activity, resulting in an inhibition of GABA release. Phosphorylation of VDCCs and K^+ channels also contributes, in a more minor way, to the action of baclofen. Direct inhibition of adenylate cyclase also inhibits GABA release. PKC activity, in contrast, has little direct effect on GABA release but we speculate that it acts via the cAMP pathway to affect both basal release and to modulate the action of baclofen. We believe these results are the first demonstration of such cross-talk in the mammalian central nervous system. As both PKC and PKA activation potentiate GABA release, such an interaction may provide a means of negative feedback to help achieve greater control over GABA release. The exact functional consequences of this cross-talk and its underlying mechanisms await further investigation.

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