Neurotensin inhibition of the hyperpolarization-activated cation current (I_h) in the rat substantia nigra pars compacta implicates the protein kinase C pathway

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1. Whole-cell patch-clamp recording was performed from principal neurones of the substantia nigra pars compacta (SNc). In 66% of these neurones, neurotensin (NT) induced, at −60 mV, an inward current associated with an increase in conductance.

2. Principal neurones displayed, in response to hyperpolarizing voltage steps, the voltage-dependent inward cationic current, I_h. This current activated at potentials more negative than −65 mV and reached a maximum at −106 ± 4 mV, with a half-activation potential of −86 ± 3 mV. Its estimated reversal potential was −43 ± 7 mV and its activation curve was fitted with two exponentials.

3. In 41% of neurones showing the inward current, NT (0·5 μM) also reversibly reduced the amplitude of I_h. The diminution was 48·5 ± 12% when voltage steps were made from −60 to −95 mV. The decrease in I_h resulted from a reduction in the maximal current with no change in the voltage dependence of activation.

4. Forskolin (10 μM), an activator of adenylate cyclase, increased I_h by shifting its activation range to more positive potentials, but it did not alter the NT inhibition of I_h.

5. The effect of NT was blocked by staurosporine (0·5 μM) and by PKC-(19–31) (0·5 μM), a specific protein kinase C (PKC) inhibitor, but was unaffected by Walsh's peptide (100 μM), a specific inhibitor of protein kinase A. The reduction of I_h was mimicked by 1-oleoyl-2-acetyl-*sn*-glycerol (0·5–10 μM), an analogue of diacylglycerol, an endogenous PKC activator.

6. These results suggest that the inhibition of I_h by NT involves a phosphorylation mechanism that implies activation of PKC.

The inward cationic rectifier current is a mixed cation current that is activated by hyperpolarization. In pacemaker neurones of the heart, this current was first described as I_f (Brown & DiFrancesco, 1980; Yanagihara, Noma & Irisawa, 1980), where it contributes to the determination of the heart rate. In neurones, the inward cationic rectifier current was first described as I_q in hippocampal pyramidal cells (Adams & Halliwell, 1981) and I_h in dorsal root ganglion (DRG) neurones (Mayer & Westbrook, 1983). Since then, it has been widely described in central neurones, where it contributes to the pattern of spike discharge (see review by Pape, 1996).

The inward cationic rectifier current is known to be modulated by cAMP. In sino-atrial cells, this current is modulated in opposite ways by noradrenaline and acetylcholine, which stimulate and inhibit adenylate cyclase, respectively, and so affect the level of cAMP (see review by DiFrancesco, 1993). cAMP modulates I_f by shifting its activation range to more positive potentials. This effect does not involve phosphorylation but rather a direct interaction of cAMP with the channels at their cytoplasmic side (DiFrancesco & Tortora, 1991). I_h is also modulated via cAMP in bull-frog sympathetic neurones (Tokasima & Akasu, 1990) and in nodose ganglion cells (Ingram & Williams, 1994). The modulation of I_h by cAMP has been demonstrated in multiple types of central neurones (Bobker & Williams, 1989; Pape & McCormick, 1989; Pedarzani & Storm, 1995). Whether the change in voltage dependence results from a cAMP-dependent protein kinase (PKA) can not be deduced unambiguously from available data (see review by Pape, 1996).

Mesencephalic dopaminergic neurones exhibit a pronounced I_h (Lacey & North, 1988; Lacey, Mercuri & North, 1989; Hainsworth, Roper, Kapoor & Ashcroft, 1991; Yung, Hausser & Jack, 1991; Jiang, Pessia & North, 1993). This current may modulate cellular excitability by limiting the amplitude and duration of prolonged hyperpolarizing events in the dopaminergic cells (Mercuri, Bonci, Calabresi, Stefani & Bernardi, 1995), which receive a strong inhibitory input, mainly from the striatum. The purpose of this study was to determine the action of neurotensin (NT) on I_h in rat substantia nigra pars compacta (SNc) neurones. In this

structure, NT binding sites occur at high density in regions that contain dopamine cell bodies (Palacios & Kuhar, 1981; Boudin, Pelaprat, Rostène & Beaudet, 1996), and this has led to speculation that this peptide may be involved in the pathophysiology of Parkinsonism (Chinaglia, Probst & Palacios, 1990). NT has been shown to increase firing of dopaminergic cells both *in vivo* and *in vitro* (Pinnock, 1985; Mercuri, Stratta, Calabresi & Bernardi, 1993). Under voltage-clamp recordings in ventral tegmental dopaminergic neurones, NT evokes an inward current resulting from an enhancement of non-selective cationic conductance and a decrease of K^+ conductance (Jiang, Pessia & North, 1994). Wu, Li & Wang (1995) have since confirmed these findings in the SNc.

Here we report that activation of NT receptors reversibly reduces I_h in SNc neurones. Our experiments suggest that this effect involves protein kinase C (PKC). Abstracts of this work have appeared previously (Cathala & Paupardin-Tritsch, 1995, 1996).

METHODS

In vitro slice preparation

Male Wistar rats, aged between 10 and 15 days, were anaesthetized with 1% halothane and decapitated. The brain was removed and placed in ice-cold bicarbonate-buffered saline (BBS) of the following composition (mM): NaCl, 125; KCl, 2·5; NaH_2PO_4, 1·25; $NaHCO_3$, 26; $CaCl_2$, 0·4; $MgCl_2$, 1; glucose, 25; saturated with 95% O_2 and 5% CO_2. The tissue was trimmed with a razor blade and fixed with cyanoacrylate glue to the chuck of a vibroslicer (DSK microslicer). The block of tissue containing the SNc was cut into coronal slices (250 μm thickness), starting from the ventral surface. The slices containing the SNc (below the pons and up to the beginning of the mammillary body) were kept. The left and right halves of the slice were transferred into an incubating chamber (volume, 80 ml), where they were perfused continuously with BBS (0·5–1 ml min^{-1}) and bubbled with 95% O_2 and 5% CO_2. The chamber was maintained at a constant temperature of 30–32 °C. The slices were allowed to equilibrate for at least 1 h before being transferred to the experimental set-up. During this time the calcium level of the BBS was increased to 2 mM. Then the slices were placed under the microscope in a chamber containing oxygenated BBS and maintained at room temperature. The bath volume was 0·5 ml with a constant flow rate of 1 ml min^{-1}. The SNc was easily identified with a Nomarski optic microscope (Nikon). Micropipettes were placed into the region under visual control.

Tight-seal whole-cell recording

Tight-seal whole-cell recordings were made using borosilicate glass pipettes pulled in two stages to a tip of about 1–2 μm o.d. The tip of the electrode was coated with wax to a length of 1 cm. The electrodes were filled with a solution containing (mM): KCl, 115; $MgCl_2$, 5; $CaCl_2$, 1; Hepes, 10; EGTA, 10; ATP, 4; GTP, 0·4; phosphocreatine, 6·8 mg ml^{-1}; creatine kinase, 50 U ml^{-1}; pH adjusted to 7·3 with KOH; tip resistance, ~2·5–3 MΩ. When chloride-free solution was required, KCl was replaced by potassium gluconate (100 mM).

Liquid junction potentials were compensated in reference to a Ag–AgCl pellet in direct contact with the bath fluid. As we never changed the external chloride concentration, liquid junction potentials remained stable during all of the experiments. The electrode was advanced into the brain slice and a seal with the cell membrane was obtained by applying negative pressure. Once a high-resistance seal (> 1 GΩ) had been established, the holding potential was set to −60 mV and the whole-cell configuration was obtained by further suction. Both capacitance and series resistance compensation were used. Only cells with capacitance transients well described by a single exponential, that is with amplitudes of any subsequent components less than 10% of that of the first exponential component, were accepted for further analysis. Values of access resistance (which were checked repeatedly during each experiment) ranged from 5 to 9 MΩ and recordings were rejected if this changed by 10%. The membrane currents were measured with a single electrode voltage-clamp amplifier (Axopatch 200A), viewed on an oscilloscope, recorded on a digital tape recorder (Biologic) and displayed on a pen recorder (Astro-med). Data were also captured on-line at a sampling rate of 5 kHz using an interface coupled to a microcomputer running the pCLAMP software (version 5.5; Axon Instruments).

Data analysis

I_h was evoked by applying hyperpolarizing voltage command steps from a holding potential of −60 mV to a potential of −95 mV. The steps were applied with an interval of 30 s and their duration was 6 s. We did not try to hyperpolarize to more negative potentials (except for tail current experiments) since it was difficult to maintain cells hyperpolarized below −100 mV. The degree of space clamp of these cells in slices is unknown, but the finding that the rate and voltage dependence of activation of the inward rectifier current were smooth and continuous functions of the membrane potentials suggests that, in this range of potentials, sufficient clamp was obtained to avoid serious errors. The amplitude of I_h was measured as the difference between the instantaneous current at the beginning of the step and the steady-state current at the end of the voltage step. Averaged data are expressed as means ± S.D. Statistical significance between data groups was tested using Student's t test. Statistical differences between groups are noted in the figures by an asterisk.

The steady-state activation curve of I_h was constructed by measuring tail currents elicited by repolarizing the membrane to holding level (typically −60 mV) following voltage steps of 6 s to between −65 and −120 mV. Because of technical limitations, we often were unable to collect data at potentials where I_h was fully activated and the maximum conductance (G_{max}) was not reached in such experiments. The current–voltage (I–V) curves were obtained by applying a series of steps from a resting potential of −60 mV to a potential of −95 mV, with increments of 5 mV. This was appropriate to use since the values obtained at a potential of −95 mV were close to G_{max}. In tracing the I–V curves, the amplitude of I_h was expressed in picoamps for data from a single cell or was normalized from −95 mV when the data were compiled from several cells.

Drugs

All the experiments were done in BBS solution containing tetrodotoxin (TTX, 0·5 μM). To reduce contamination of I_h tail currents by other currents, tail experiments were performed in the presence of 4-aminopyridine (1 mM), barium (300 μM), TEA (100 μM) and cadmium (50 μM). Drugs were applied by adding them at known concentrations (indicated in the text) to the BBS. Protein kinase inhibitor peptides were applied through the patch pipettes. Peptide PKC-(19–31) or Walsh's peptide was prepared from a stock solution by adding to the internal solution. After the rupture of the patch, a period of 10–15 min was allowed for the peptide to enter the cell before starting the experiment. The following drugs were

used: TTX (Sigma and Latoxan), NT (Peninsula Laboratories), forskolin (Sigma), staurosporine (Sigma), PKC-(19–31) (Boehringer), Walsh's peptide (Sigma), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; Sigma), ZD7288 (Tocris Cookson, Bristol, UK). All the salts and proteins were obtained from Sigma.

RESULTS

Whole-cell recordings were made from neurones that exhibited the electrophysiological properties of the principal neurones of the SNc, presumably dopaminergic cells (Lacey *et al.* 1989; Yung *et al.* 1991). Recordings were done in the central part of the SNc. Secondary neurones, occasionally recorded, were not taken into account in this study.

NT evokes an inward current

In cells clamped at −60 mV, a 2 min bath application of NT (0·5 μM) induced, in 66% of the neurones ($n = 108$), an inward current (Fig. 1). On average, the NT-induced inward current reached a maximum in 40 s, began to decline while the drug application continued and returned to baseline level within 2 min. Membrane conductance was measured periodically by applying pulses of 30 ms duration from a potential of −60 to −70 mV (not shown) or by measuring the instantaneous current during the I_h protocols (see Methods section). The current induced by NT was associated with a small increase (on average < 10%) in instantaneous conductance (see Fig. 3*A*) and an increase in noise that persisted several minutes after wash (Fig. 1). The conductance underlying this inward current is probably the non-selective cationic conductance described in the basal forebrain (Farkas, Nakajima & Nakajima, 1994), in the ventral tegmental area (Jiang *et al.* 1994; Farkas, Chien, Nakajima & Nakajima, 1996), as well as in the SNc (Pinnock, 1985; Mercuri, Stratt, Calabresi & Bernardi, 1993). This current was unaffected by TTX (0·5 mM), forskolin (10 μM; $n = 8$), staurosporine (0·5 μM; $n = 10$), OAG (0·25–10 μM; $n = 12$), PKC-(19–31) (0·5 μM; $n = 16$) and Walsh's peptide (100 μM; $n = 11$; data not shown). These results indicate that the increase in cationic conductance induced by NT in the SNc does not involve either PKA or PKC.

I_h properties

Figure 2*A* illustrates a typical family of slowly activating inward currents evoked by hyperpolarizing voltage steps. In this experiment, the neurone was clamped at a holding potential of −60 mV and stepped from −65 to −120 mV. These currents were observed in 96% of the recorded neurones ($n = 124$). The instantaneous component was followed by an inward current that developed over several seconds. The slowly developing component increased in amplitude with the size of the hyperpolarizing command. The current amplitude did vary between cells. The average amplitude was 166 ± 58 pA at −95 mV ($n = 35$).

This current was insensitive to cadmium (100 μM) and barium (300 μM) and persisted in chloride-free internal solution (data not shown). It was fully blocked by 3 mM external caesium ($n = 4$; Fig. 2*B*) and also completely suppressed by 10 μM ZD7288 ($n = 4$; Fig. 2*C*), a drug described as a specific blocker of the I_h current (Harris & Constanti, 1995). These properties are consistent with the pharmacological properties of the I_h current, a cationic current described as a specific conductance of the principal cells of the SNc (Mercuri *et al.* 1995).

I_h current is characterized by slow activation. I_h showed no sign of inactivation during maintained hyperpolarization up to 30 s duration (data not shown). When the cell was hyperpolarized at −95 mV, the pulse had often not reached the steady state at 6 s and longer steps were used to determine the rate of activation. The current activation was best described by the sum of two exponentials. The time constants of activation τ_1 and τ_2 were clearly voltage dependent, decreasing with hyperpolarization. Pooled activation time constants give values for τ_1 and τ_2, respectively, of 1·51 ± 0·445 and 9·55 ± 3 s at −90 mV ($n = 11$) decreasing to 0·7 ± 0·4 and 4·83 ± 2 s at −110 mV ($n = 4$).

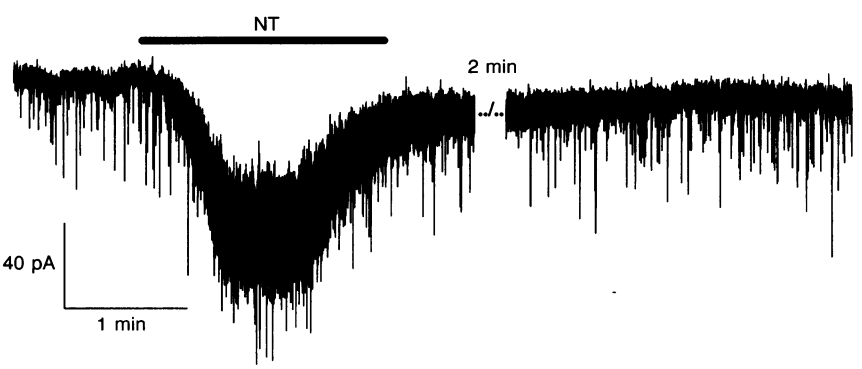

Figure 1. A 2 min bath application of neurotensin (NT; 0·5 μM) induces, at a membrane potential of −60 mV, an inward current associated with an increase in noise

The baseline returns to the control level during the application of NT (indicated by horizontal bar). Rapid inward deflections were due to spontaneous activity.

The instantaneous current jump at the end of the voltage step was larger than at the beginning, indicating that the total membrane conductance increased during the voltage step. Thus, the increase during the step was due to the activation of an inward current and not the deactivation of an outward current. Inward tail current reflecting the conductance activated during the voltage steps were apparent after the steps and decayed back to baseline as the conductance deactivated. The tail current amplitudes were normalized to the maximal amplitude and plotted against the membrane potential to which the neurone was stepped during activation of I_h (Fig. 2*D*). The tail amplitude saturated at large hyperpolarized potentials, as the conductance approached G_{max}. The resulting data were well fitted by the Boltzmann equation:

$$I/I_{max} = \{1 + \exp[(V_m - V_{½})/k]\}^{-1},$$

where I_{max} is the maximum current, I is the amplitude of the tail current at the beginning of I_h deactivation, V_m is the membrane potential, $V_{½}$ is the membrane potential at which I_h is half-activated and k *is* the slope factor. I_h was activated at potentials more negative than −65 mV and reached a maximum around −105 mV (Fig. 2*D*). Data pooled from fifteen cells give a value for V_{max} of −106 ± 4 mV. Boltzmann parameters derived from the fit give values for $V_{½}$ and k, respectively, of −86 ± 3 mV and 6 ± 1 ($n = 15$).

Since other currents were activated at the potential at which I_h appeared to reverse ($V_{rev,h}$) we could not determine $V_{rev,h}$ directly. Instead we evaluated $V_{rev,h}$ following Mayer & Westbrook (1983) by determining the intersection of the instantaneous *I–V* relationships recorded at the resting membrane potential (where the conductance underlying I_h, G_h, is not activated) and at −95 mV (where G_h is strongly activated). The instantaneous *I–V* plots were linear in both cases, and the extrapolated lines intersected at a potential of −43 ± 7 mV ($n = 5$; range, −32·5 to −54 mV). This method is subject to the considerable errors involved in such extrapolation. Nevertheless, this value of −43 mV is close to the value reported by others (see review by Pape, 1996). This value was used to compute G_h from $I_h = G_h(V - V_{rev,h})$. G_{max} was 1·56 ± 0·6 nS ($n = 15$).

Inhibition of the I_h current by NT

NT inhibits I_h in addition to inducing an inward current. Figure 3 illustrates the effect of NT (0·5 μM) on I_h. In the graph of Fig. 3*A*, I_h amplitude and the instantaneous current measured at the beginning of the pulse elicited by

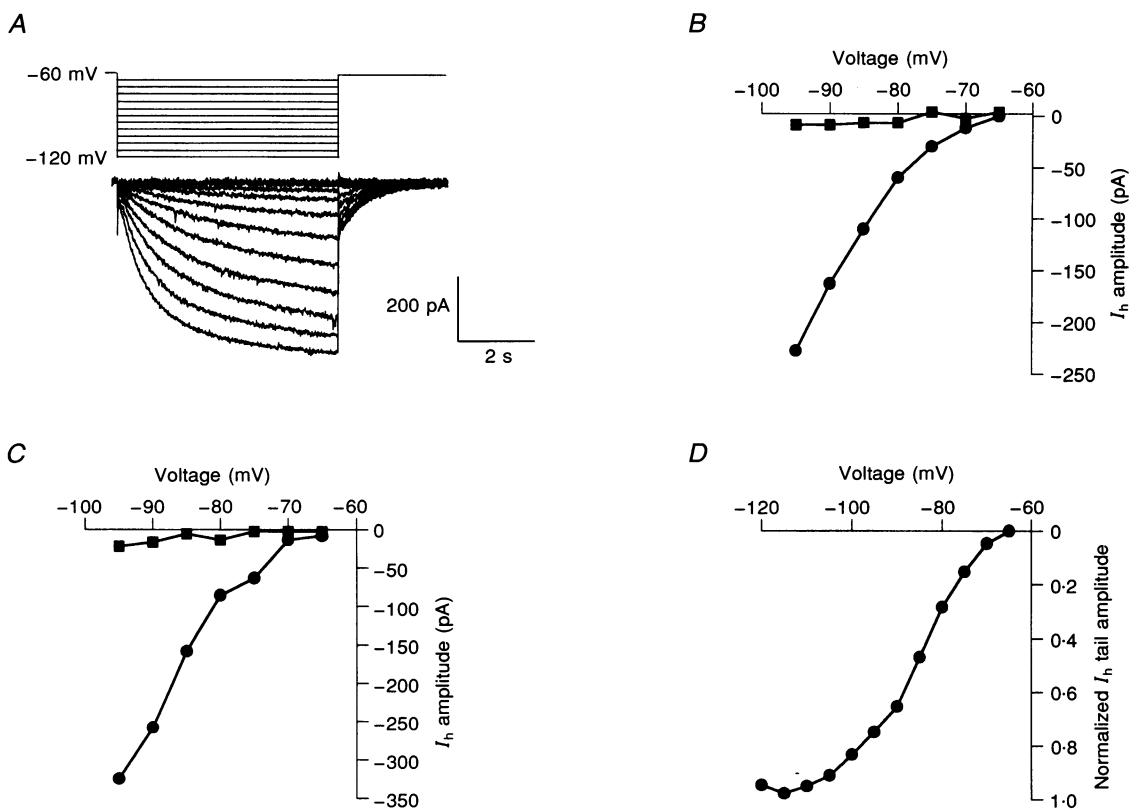

Figure 2. I_h properties

A, I_h currents evoked by command pulses from a holding potential of −60 mV to −120 mV in increments of 5 mV. *B*, action of external caesium on I_h. *I–V* curves were plotted in the absence (●) or in the presence of caesium (■; 3 mM). *C*, action of ZD7288 on I_h. *I–V* curves were plotted in the absence (●) or in the presence of ZD7288 (■; 10 μM). *D*, tail *I–V* plot. I_h activated below −65 mV and reached a maximum around −105 mV ($n = 15$).

voltage steps from −60 to −95 mV were plotted *versus* time. It shows records from a neurone where application of NT reduced by 40% the amplitude of I_h. This decrease appeared with a 60 s latency, lasted 4–5 min and was reversed by the wash-out of NT. This reduction in I_h current amplitude could be reproduced several times, without desensitization, and was observed in 41% of recorded neurones showing the increase in conductance ($n = 17$).

All the neurones in which I_h was decreased also responded to NT with an inward current. However, the I_h reduction was not due to a space-clamp artefact since the effect of NT on I_h and the inward current described in Fig. 1 did not have the same time course. Moreover, the effect of NT on I_h was maintained during longer applications in contrast to the inward current (Fig. 3*A*). Furthermore, the I_h inhibition was not observed in all the neurones showing the inward current but observed in 41% of these cells. To reduce potential artefacts, the traces, the *I–V* curves shown in the present paper as well as the collected values for statistical analysis were always obtained when the baseline had returned to the level control, at least 2–3 min after beginning the NT application.

I–V curves (Fig. 3*B*) as well as tail *I–V* curves (Fig. 3*C*) showed that NT decreased I_h at all potentials tested. The average reduction in I_h amplitude obtained with steps to −95 mV was 48·5 ± 12% ($P < 0{\cdot}001$). The activation curve from I_h tail amplitude measurements was fitted by a Boltzmann function to estimate $V_{½}$ and G_{max} (Fig. 3*B*). NT

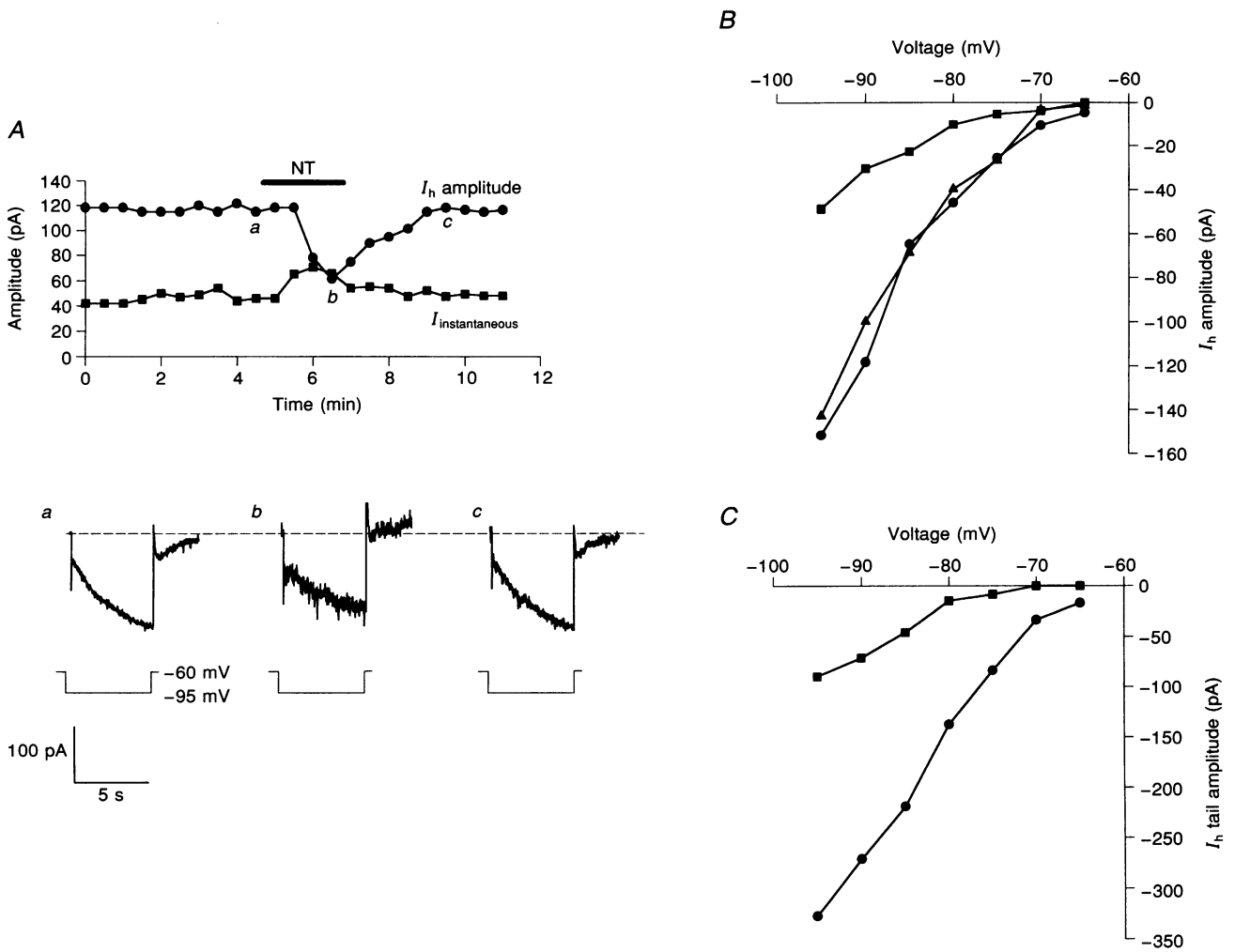

Figure 3. NT inhibits I_h

A, the effect of NT application (shown by horizontal bar) on the amplitude of I_h current (●) and the instantaneous current at the beginning of the pulse (■) is plotted against time. Current responses were obtained following hyperpolarizing steps (from −60 to −95 mV, 6 s duration, 30 s interval) before (*a*), during (*b*) and after (*c*) a 2 min bath application of NT (0·5 μM). Representative traces (*a*, *b* and *c*) elicited by the hyperpolarizing steps are displayed below. The increase of instantaneous current observed at the beginning of the pulse (*b*) is due to the increase in membrane conductance. *B*, *I–V* plot of the effect of NT on I_h (from −60 to −95 mV, in increments of 5 mV). ●, control; ■, NT; ▲, recovery. *C*, tail *I–V* plot of the effect of NT. ●, control; ■, NT.

did not affect $V_{½}$ (shift from −83 mV to −86 mV); however, I_{max} was markedly reduced (shift from 370 to 100 pA), which corresponded to a reduction in G_{max} of 70%. Similar results were obtained from activation curves derived from I_h amplitude analysis where NT did not significantly change $V_{½}$ (shift from −90·33 ± 2 to −92·33 ± 6 mV; $n = 3$; $P = 0·51$). In contrast, it did cause a significant reduction in G_{max} of 44 ± 12% ($n = 3$; $P < 0·002$). We conclude that NT acted to reduce the maximal current with no apparent change in the voltage dependence of I_h.

cAMP is not implicated in NT inhibition of I_h

Both cardiac and neuronal I_h are known to be modulated by cAMP. A decrease in the intracellular concentration of cAMP shifts the activation curve to more hyperpolarized potentials (DiFrancesco *et al.* 1991). Inversely, an increase in cAMP facilitates the activation of I_h. To assess the role of cAMP in the NT inhibition of I_h, forskolin, an activator of the adenylate cyclase was used to test whether increasing intracellular cAMP would alter the effect of NT on I_h. Forskolin (10 μM) was first applied to the bath during 10 min to allow sufficient time for the drug to diffuse within the cell. As in cardiac cells (DiFrancesco, 1993) and in dopaminergic neurones (Jiang *et al.* 1993), we noted (Fig. 4*A*) that forskolin increased the amplitude of I_h by 25 ± 5% (at −95 mV). This increase resulted from a positive shift of the *I–V* curve; $V_{½}$ was changed by 5·33 ± 0·4 mV (from −91·6 ± 1 to −86 ± 2 mV; $n = 3$; $P < 0·001$).

Unexpectedly, we found that NT could still inhibit I_h in the presence of forskolin (Fig. 4*B*). The inhibition was 46 ± 4·7% at −95 mV ($P < 0·001$) and occurred in 37·5% of the neurones ($n = 8$). This value was not significantly different from the diminution observed in the absence of forskolin ($P = 0·71$). These results indicate that cAMP is not implicated in the NT inhibition of I_h as neither the percentage of cells in which NT reduced I_h nor the magnitude of its reduction were affected by forskolin.

The PKC pathway is implicated in the modulation of the I_h current by NT

Activation of NT receptors in the SNc has been shown to stimulate phospholipase C (PLC) (Snider, Forray, Pfenning & Richleson, 1986; Hermans, Maloteaux & Octave, 1992) yielding inositol 1,3,4-trisphosphate (IP_3), which induces the release of calcium, and diacylglycerol (DAG), which activates PKC. Thus, I_h modulation by NT could occur through activation of PKC.

In order to test the role of the PKC, two different PKC inhibitors staurosporine and a specific pseudosubstrate inhibitor of PKC, PKC-(19–31) (House & Kemp, 1987) were used. First, cells were pretreated for 40 min with staurosporine (0·5 μM) applied exogenously. Staurosporine is a potent, although non-specific, PKC inhibitor (Ruegg & Burgess, 1989). Staurosporine itself did not affect the amplitude of the I_h current. Indeed, no statistical difference was obtained between the I_h amplitude measured in the absence (158·71 ± 59 pA; $n = 18$) or in the presence of staurosporine (157·77 ± 52 pA; $n = 18$; $P = 0·14$). However, the effect of NT on I_h was totally abolished ($n = 9$; Fig. 5*Ba* and *C*). Since NT reduced I_h current in 41% of neurones showing the inward current, an effect might have been expected in four of the nine cells. We concluded that staurosporine blocked the NT effect on I_h. A similar effect was observed with PKC-(19–31). This peptide (0·5 μM) was applied in the pipette solution. After disruption of the membrane, a period of 15 min was allowed to enable the peptide to diffuse within the cell. The I_h amplitude measured 15 min after disruption of the membrane (148 ± 55 pA;

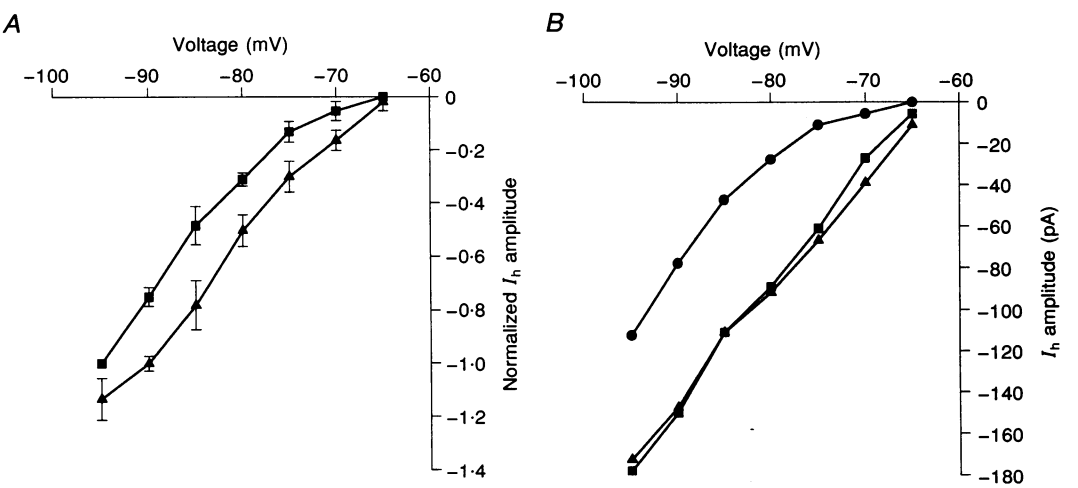

Figure 4. Forskolin does not alter the NT inhibition of I_h

A, an *I–V* plot of I_h in the absence (control, ■) or presence of forskolin (▲; 10 μM). The amplitude is normalized from −95 mV in control conditions. *B*, *I–V* plot of the inhibition of I_h by NT (0·5 μM) in the presence of FK. ■, control, FK alone; ●, FK + NT; ▲, recovery, FK alone.

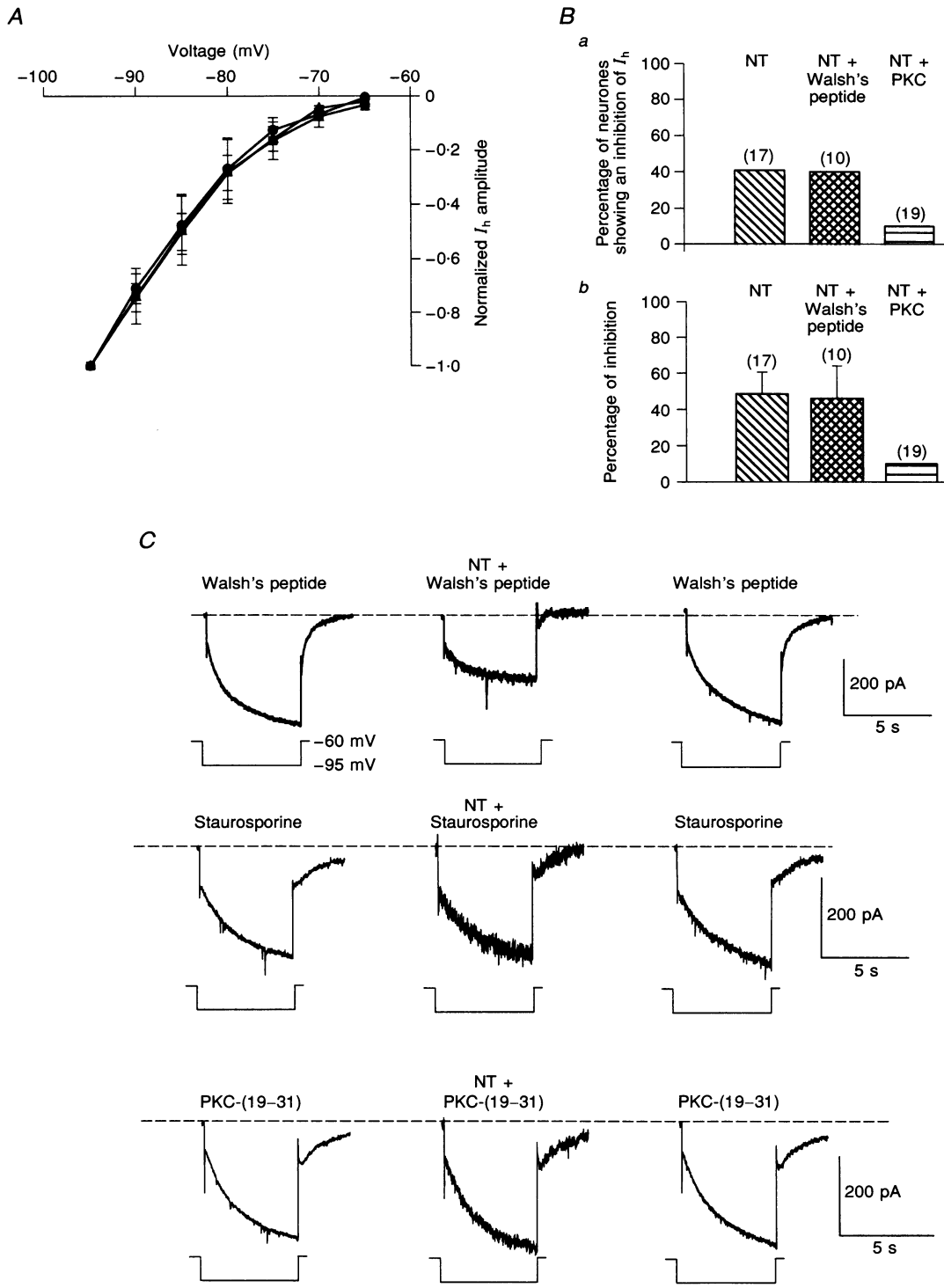

Figure 5. NT inhibition of I_h is selectively blocked by PKC inhibitors

A, *I–V* plot of the effect of PKC-(19–31) (●; $n = 3$) and of Walsh's peptide (■; $n = 16$) on the voltage dependence of the activation of I_h compared with the control condition (▲; $n = 15$). *B*, effect of NT (0·5 μM) on I_h in the absence ($n = 7$ out of 17) or the presence of PKC-(19–31) (0·5 μM; $n = 2$ out of 19), Walsh's peptide (100 μM; $n = 4$ out of 10) or staurosporine (Stauro; 0·5 μM; $n = 0$ out of 9). *a* illustrates the percentage of cells showing an I_h inhibition in response to application of NT. Numbers in parentheses are the number of cells tested. *b* illustrates the percentage of I_h amplitude inhibition observed in the different experimental conditions. Average data are expressed as means ± S.D. *C*, representative traces elicited by hyperpolarizing steps from −60 to −95 mV are displayed before, during and after application of NT, in the presence of these three compounds.

$n = 16$) did not differ from I_h amplitude measured in identical conditions without the peptide in the pipette (152·1 ± 55 pA; $n = 19$; $P = 0·8$). Moreover, the current–voltage relationship was not affected by PKC-(19–31) ($n = 3$; Fig. 5*A*). With PKC-(19–31) in the pipette, NT induced a small decrease (10% instead of the 48·5% expected; Fig. 5*Bb*) of the I_h current in only 10% (instead of the 41% expected) of the cells ($n = 19$; Fig. 5*Ba* and *C*). These results indicate that PKC-(19–31) blocks qualitatively and quantitatively the NT induced I_h inhibition.

Inhibition of I_h was specific to PKC to the extent that the results obtained with the PKC inhibitors were not reproduced with Walsh's peptide, a specific inhibitor of the cAMP-dependent PKA. This inhibitor was applied in the pipette solution at a saturating concentration of 100 μM and cells were dialysed for 15 min before NT was applied. Walsh's peptide did not affect the amplitude of the I_h current. The I_h amplitude measured 15 min after disruption of the membrane (152·1 ± 51 pA; $n = 19$) did not differ from I_h amplitude measured in identical conditions without the peptide in the pipette (152·1 ± 55 pA; $n = 19$; $P = 0·21$). Moreover, the *I–V* relationship is not affected by Walsh's peptide ($n = 16$; Fig. 5*A*). In the presence of Walsh's peptide, application of NT still reduced the amplitude of I_h (Fig. 5*Bb* and *C*). The inhibition at −95 mV was 46 ± 18% ($P < 0·01$) and not significantly different from the inhibition observed in the absence of Walsh's peptide ($P = 0·76$). This effect was observed in 40% of the cells ($n = 10$; Fig. 5*Ba*).

To confirm the involvement of PKC in the signalling pathway mediating the effects of NT, we studied the effects of OAG, an analogue of DAG. OAG was applied to the bath in a range of concentrations from 0·5 to 10 μM. In 23% of the cells ($n = 13$) OAG caused a significant decrease (47 ± 8%) in the amplitude of the I_h current ($P < 0·005$; Fig. 6). The low percentage of cells responding may come from the limited accessibility of OAG to the cells. Indeed, OAG is a unstable and lipophilic compound. These results suggest that PKC is involved in the modulation of I_h by NT since specific PKC inhibitors block the NT-mediated effect and OAG mimics its effect.

DISCUSSION

NT induces an inward current

NT is a neuropeptide released from afferent GABAergic fibres onto the dopaminergic cells of the SNc where NT binding sites are found in high density (Palacios & Kuhar, 1981; Boudin *et al.* 1996). In response to NT, 66% of the SNc neurones, developed an inward current associated with a small increase in conductance and a large increase in noise that persisted after wash. This result is consistent with previous studies in the SNc and the ventral tegmental area (VTA) where NT induced an inward current dependent on change in a mixed cationic conductance (Jiang *et al.* 1994; Wu *et al.* 1995; Farkas *et al.* 1996). Jiang *et al.* (1994) and Wu *et al.* (1995) also reported that this inward current is also dependent on a decrease in a potassium conductance which overlapped with the predominant increase in cationic conductance. We only observed an increase in conductance but this does not exclude the possibility of a decrease in potassium conductance hidden by the increase in cationic conductance. Indeed, at a potential of −60 mV and in the recording conditions identical to ours, Jiang *et al.* (1994), as well as Wu *et al.* (1995), only detected the increase in cationic conductance. The reduction in potassium conductance was

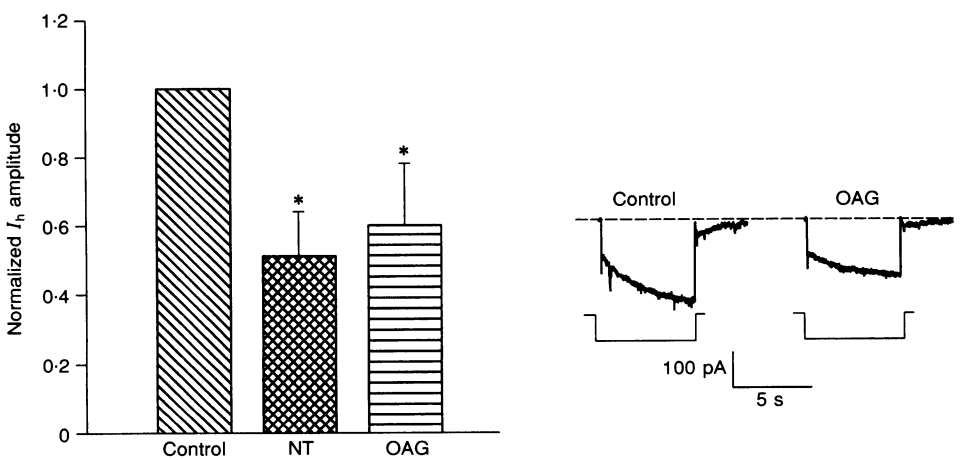

Figure 6. NT inhibition of I_h is mimicked by OAG, a DAG analogue

Effect of OAG (0·5–10 μM; $n = 3$) compared with the effect of NT (0·5 μM; $n = 7$) on the normalized amplitude of I_h. Representative traces elicited by the hyperpolarizing steps from −60 to −95 mV are displayed on the right, in the absence (Control) or the presence of OAG (1 μM). We observed that OAG did not increase the instantaneous current. Average data are expressed as means ± S.D. Statistical difference from the control (*) was tested with Student's *t* test.

observed when the NT effect was tested below the equilibrium potential for potassium (Jiang *et al.* 1994) or in sodium-free solution (Wu *et al.* 1995).

Activation of the NT inward current did not involve cAMP, cGMP, or internal Ca^{2+} (Farkas *et al.* 1996). We observed that this inward current was not affected by inhibition of PKC and PKA, suggesting that the channel mediating this increase in conductance is not regulated by a conventional phosphorylation/dephosphorylation mechanism. Using the outside-out configuration of the patch clamp, Chien, Farkas, Nakajima & Nakajima (1996) recorded single cation channel openings induced by NT, suggesting that at least a part of the response was mediated by a G protein.

I_h properties

Principal neurones of the SNc exhibit I_h, a mixed cationic current activated by hyperpolarization. The I_h current that we recorded in these neurones had similar characteristics to those described in other cells types as well as dopaminergic cells of the mesencephalon.

We have shown that I_h is blocked by external caesium and low concentration of ZD7288, a drug described as a specific blocker of I_h. The reversal potential (−43 mV), the activation range (between −65 and −105 mV), the maximal conductance (1·6 nS), and the half-activation potential (−86 mV) are all close to values reported previously (see review of Pape, 1996). The major difference is the particularly slow rate of activation. Several seconds are required to reach the steady state. The current kinetics are described by two exponentials, with slow time constants. Although multiexponential kinetics have already been described in other cell types (Banks, Pearce & Smith, 1993; Solomon & Nerbonne, 1993; Budde, White & Kay, 1994), previous works on VTA and SNc neurones shows single exponential kinetics as well as shorter time constants (Jiang *et al.* 1994; Mercuri *et al.* 1995; Watts, Williams & Henderson, 1996). Such slow kinetics might be explained by a temperature dependence of activation since our recordings were made at room temperature. Developmental factors might also be involved since our experiment were performed on younger rats. Furthermore, since the gene or genes that encode the I_h channel has not yet been identified, it is difficult to discriminate between multiple gating processes of the same population of channels or the existence of two distinct populations of I_h channels.

NT modulation of I_h

Here, we show that NT inhibits I_h reversibly in a sub-population of SNC neurones.

Watts *et al.* (1996) have recently shown that the inhibition of I_h by baclofen in rat SNc neurones may be secondary to potassium current activation. Similarly, NT inhibition of I_h that we observed could have been the result of the increase in membrane conductance. However, we can exclude this hypothesis since (1) the two effects do not have the same time course, and (2) I_h inhibition was observed only in 41% of the cells showing the increase of conductance. Activation of cationic conductance and inhibition of I_h are obviously not correlated.

In many neuronal and non-neuronal cell types, modulation of I_h results from changes in the level of cyclic AMP (see review of Pape, 1996). Changes in I_f (in heart) or I_h (in neurones) result from a shift in voltage dependence rather than a change in maximal current. Activation of adenylate cyclase will increase intracellular cAMP, and so shift I_h activation to more positive potentials.

The displacement of activation for I_h that we observed with forskolin in the SNc was consistent with these observations. However, the mechanism by which NT inhibits I_h does not seem to involve cAMP. First, NT modulation of I_h is not altered in the presence of forskolin. Second, the inhibition of I_h results from a decrease in the maximal current with no change in the voltage dependence.

NT acts through a PKC pathway

Activation of NT receptors stimulates PLC in the SNc (Wu *et al.* 1995; Wu & Wang, 1995) as well as in other cell types (Snider *et al.* 1986; Hermans *et al.* 1992). The phosphoinositide hydrolysis produces two second messengers, IP_3 and DAG, an activator of PKC.

Our experiments suggest that PKC mediates the NT effect on I_h. First, NT inhibition of I_h was blocked by staurosporine and the specific PKC inhibitor, PKC-(19–31) peptide. Second, the Walsh's peptide, a specific inhibitor of PKA, did not affect the reduction in I_h amplitude induced by NT. Third, a PKC activator (OAG) mimicked the NT-induced inhibition of I_h. We found that OAG reproduced to some extent (23% of the cells tested) the results obtained with NT. Possibly OAG had only a limited access to our cells since it is a lipophilic and unstable compound, rapidly converted to an inactive form and easily trapped in surrounding tissues.

Nevertheless, from these results, we can conclude that the NT inhibition of I_h amplitude is mediated by PKC. It is the first report that PKC can modulate I_h.

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