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NIFEDIPINE- AND ω-CONOTOXIN-SENSITIVE Ca^{2+} CONDUCTANCES IN GUINEA-PIG SUBSTANTIA NIGRA PARS COMPACTA NEURONES

BY S. NEDERGAARD, J. A. FLATMAN AND I. ENGBERG
From the Institute of Physiology, Aarhus University, DK-8000 Aarhus C, Denmark

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SUMMARY

1. The membrane properties of substantia nigra pars compacta (SNc) neurones were recorded in guinea-pig *in vitro* brain slices.

2. In the presence of tetrodotoxin (TTX) a Ca^{2+}-dependent slow oscillatory potential (SOP) was generated. Application of 0·5–20 μM nifedipine abolished both spontaneous and evoked SOPs. A tetraethylammonium chloride (TEA)-promoted high-threshold Ca^{2+} spike (HTS) was little affected by nifedipine. On the other hand, ω-conotoxin applied either locally or via the perfusion medium (1–10 μM) blocked a part of the HTS, but it did not alter the SOP.

3. In normal medium nifedipine blocked the spontaneous discharge, decreased the interspike interval (ISI) recorded during depolarizing current injections and selectively reduced the slow component of the spike after-hyperpolarization (AHP). ω-Conotoxin decreased both the rising and falling slopes of the normal action potential, reduced the peak amplitude of the spike AHP, and, in some of the neurones, reduced the ISI during depolarization. The Na^+ spikes recorded in Ca^{2+}-free medium were not altered by ω-conotoxin.

4. The SOP was not blocked by octanol (100–200 μM), amiloride (100–250 μM), or Ni^{2+} (100–300 μM). However, at 500 μM Ni^{2+} attenuated the SOP.

5. Application of apamin (0·5–2·0 μM) induced irregular firing or bursting, abolished the slow component of the spike AHP and reduced its peak amplitude. In the presence of TTX and apamin long-duration plateau potentials occurred, which were subsequently blocked by nifedipine.

6. In Ca^{2+}-free, Co^{2+}-containing medium TTX-sensitive spikes and voltage plateaux were generated by depolarizing current pulses. It is suggested that a persistent Na^+ conductance underlies the plateaux, which may be co-activated during the SOP.

7. The results suggest that the Ca^{2+} currents underlying the SOP and the HTS are different and that they activate at least two Ca^{2+}-dependent K^+ conductances. These conductances play major roles in the maintenance of spontaneous discharge and in control of membrane excitability.

INTRODUCTION

Neurones located within the pars compacta region of the substantia nigra (SNc) have been studied extensively *in vitro*, and several types of cells with different

membrane characteristics have been described (Llinás, Greenfield & Jahnsen, 1984; Kita, Kita & Kitai, 1986; Matsuda, Fujimura & Yoshida, 1987; Grace & Onn, 1989; Lacey, Mercuri & North, 1989; Silva, Pechura & Barker, 1990; Yung, Häusser & Jack, 1991; Hainsworth, Röper, Kapoor & Ashcroft, 1991). One electrophysiologically distinct neuronal type has been shown to contain dopamine (Grace & Onn, 1989; Yung *et al.* 1991). These identified dopaminergic neurones are distinguished by a slow, pacemaker-like discharge which usually occurs spontaneously during recordings in brain slices. Although the exact ionic mechanisms are not fully understood, it is believed that Ca^{2+}-dependent processes are involved in the generation and maintenance of this rhythmic firing pattern (Harris, Webb & Greenfield, 1989; Yung *et al.* 1991; Shepard & Bunney, 1991; Grace, 1991). In a recent report (Nedergaard & Greenfield, 1992) it has been suggested that two different Ca^{2+} conductances, which underlie a high-threshold Ca^{2+} spike (HTS) and an autogenous slow oscillatory potential (SOP), each play specific roles in the generation of action potentials and after-potentials in these SNc neurones. The aim of the present study was to investigate the nature and function of each of these Ca^{2+} conductances by the use of specific Ca^{2+} channel antagonists. Preliminary data from these experiments have been presented in abstract form (Nedergaard & Flatman, 1992).

METHODS

Brain slice preparation

Albino guinea-pigs of either sex (250–350 g) were anaesthetized with chloroform and decapitated. The brain was quickly removed, and a block of tissue containing the midbrain was transferred to a cooled Hepes solution containing (mM): NaCl, 120; KCl, 2·0; KH_2PO_4, 1·25; $MgSO_4$, 2·0; $CaCl_2$, 2·0; $NaHCO_3$, 20; Hepes acid, 6·7; Hepes salt, 2·6; glucose 10. Four to six coronal slices (400 μm) were cut and transferred to separate vials and stored in a Hepes solution (bubbled with 95% O_2–5% CO_2 at room temperature) for at least 2 h. In the recording chamber slices lay on a piece of lens tissue placed over a nylon mesh. The slice surface was at the interface between an atmosphere of 95% O_2–5% CO_2, saturated with water vapour at 32·5 °C, and a standard perfusion solution containing (mM): NaCl, 132; KCl, 1·8; KH_2PO_4, 1·25; $MgSO_4$, 1·3; $CaCl_2$, 2·4; $NaHCO_3$, 20; glucose, 10. The flow rate was 1·5 ml min^{-1}.

Intracellular recording and data analysis

Recordings were made using glass microelectrodes filled with 4 M potassium acetate (resistance, 45–90 MΩ), and placed in a micromanipulator. Signals were fed into a conventional bridge amplifier, digitized, and stored on videotape. Data analyses were performed on a computer employing SIGAVG software (CED, Cambridge, UK). Spike heights and durations were measured as described previously (Nedergaard & Greenfield, 1992). Membrane input resistances were calculated by measuring the steady-state voltage deflections during 0·1–0·2 nA hyperpolarizing current injections. All averaged values presented below are means ± standard deviation.

Drug solutions

The following drugs were applied to the perfusion solution in the concentrations indicated: tetrodotoxin (TTX; Sigma, Poole, Dorset; added from a reservoir in 1 ml doses (50 μM), repeated every 1 h of experiment), tetraethylammonium chloride (TEA; 5 mM; Sigma), nifedipine (0·5–20 μM; Sigma), amiloride (100–250 μM; Sigma), octanol (100–200 μM; Riedel-deHäen, Seelze, Germany), ω-conotoxin GVIA (1–10 μM; Calbiochem, San Diego, CA, USA and Research Biochemicals Inc., Natick, MA, USA), apamin (0·5–2 μM; Sigma), nickel chloride (100–500 μM; Sigma), and cobalt chloride (2·4 mM; Sigma). Nifedipine was dissolved in dimethyl sulphoxide in a stock solution (50 mM) and stored in the dark at +4 °C for maximally 36 h before use. In some experiments ω-conotoxin was applied to the slice surface near the recording site via droplets (1–5;

containing 50 μM ω-conotoxin in saline) from the tip of a broken microelectrode (4 μm outer diameter). Control experiments showed no effects of the solvents used. $CoCl_2$ was applied via a perfusion solution from which Ca^{2+} and phosphate ions were omitted.

RESULTS

Cell membrane properties recorded in normal medium

Intracellular recordings were obtained from seventy-four neurones, located in the SNc at the level of the accessory optic tract. A total of sixty-two were retained for further analysis on the basis of the following selection criteria; action potential amplitude of more than 45 mV beyond the threshold, ability to discharge repetitively in response to depolarizing current pulses lasting 1 s or more, and stable recording for at least 30 min. All these neurones had membrane characteristics similar to those of identified dopaminergic neurones *in vitro* (Grace & Onn, 1989; Yung *et al.* 1991). The mean action potential height and duration were on average $58{\cdot}7 \pm 6{\cdot}6$ mV and $2{\cdot}1 \pm 0{\cdot}5$ ms respectively. Membrane input resistances varied between 42 and 420 MΩ (mean, 96 ± 53 MΩ).

After recovery from impalement fifty-one of the sixty-two cells generated fast action potentials spontaneously at a regular rate ($2{\cdot}9 \pm 1{\cdot}8$ Hz). The remaining eleven cells displayed a steady membrane potential, negative to the firing threshold. Individual action potentials were triggered at a threshold of about -40 mV, and, in most cells they were followed by a clearly biphasic after-hyperpolarization (AHP) composed of an initial, fast component with a large amplitude, and a late, slow component which normally lasted 100–200 ms (Fig. 1*A* and *B*).

Injection of a strong hyperpolarizing current pulse resulted in the following responses, which were found in all the neurones tested: a slow, time-dependent depolarization during the pulse, and a delayed repolarization after termination of the pulse (Fig. 1*C*). Action potentials evoked by a sustained depolarization displayed frequency adaptation in most of the neurones tested (Fig. 1*D*). After termination of a depolarizing current pulse an AHP was observed, which could last several hundreds of milliseconds. Its amplitude and duration was proportional to both the strength (Fig. 1*E*) and the duration of the preceding current pulse. This response is subsequently referred to as the 'post-pulse AHP'.

Blockade of the slow oscillatory potential by nifedipine

After blockade of the fast action potential by application of TTX, underlying rhythmic fluctuations in membrane potential were revealed. These slow oscillatory potentials (SOPs) occurred spontaneously in a high proportion of the neurones, and were characterized by a slow depolarizing phase (which usually exceeded -40 mV) followed by a relatively faster hyperpolarizing phase (Fig. 2*A* and *B*). The SOP has previously been identified in SNc neurones, and shown to be Ca^{2+} dependent (Fujimura & Matsuda, 1989; Yung *et al.* 1991; Nedergaard & Greenfield, 1992). In neurones which displayed a high membrane potential (-60 mV or more) the SOPs were absent, but they could easily be evoked by a brief depolarizing pulse.

In order to determine whether a single type of Ca^{2+} channel is involved in the generation of the SOP, the effects of specific Ca^{2+} channel blockers were investigated. Application of the dihydropyridine blocker nifedipine (0·5–20 μM) to the bath was

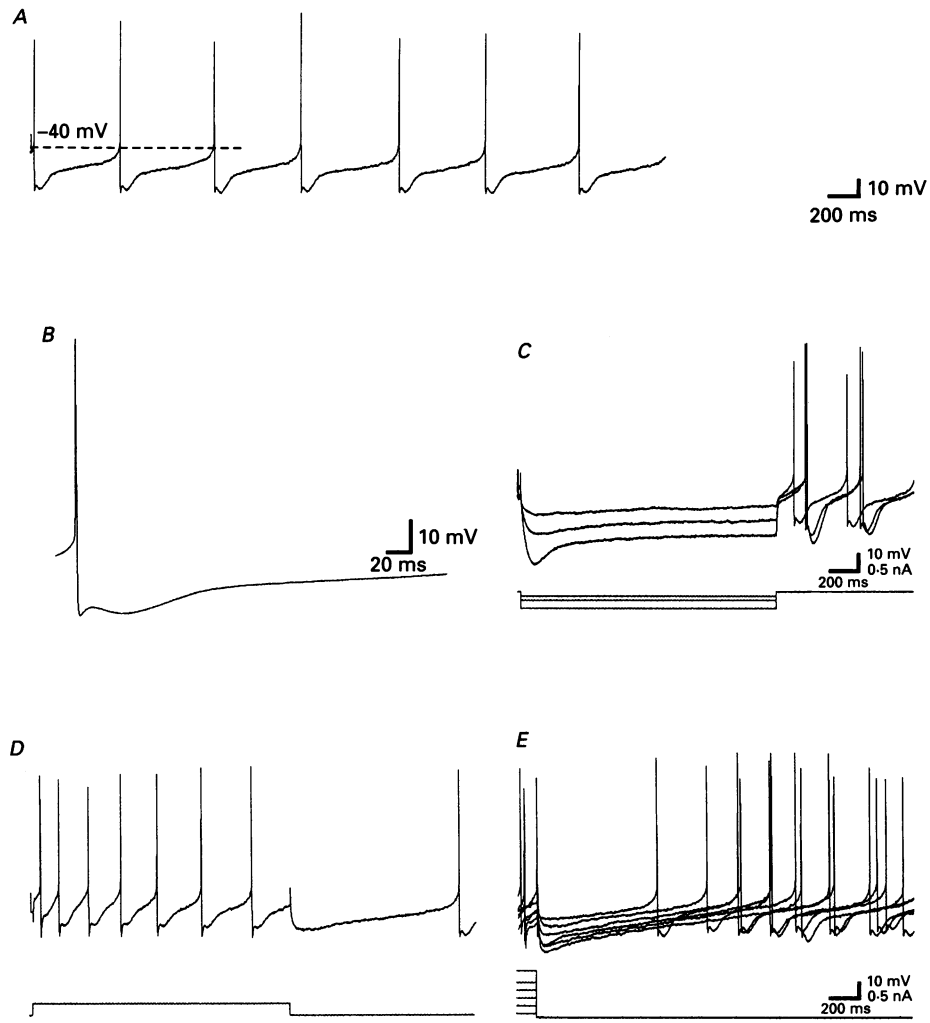

Fig. 1. Membrane characteristics of an SNc neurone. *A*, discharge of spontaneous action potentials during passive recording. Note the highly regular firing frequency. Dashed line indicates the firing threshold of −40 mV. Spike size variation in this and the following figures is due to sampling artifact. *B*, record showing the average of ten spontaneous action potentials. The spike AHP consists of an initial, fast and a late, slow component. *C*, superimposed recordings of voltage responses to the injection of hyperpolarizing current pulses (in this and the following figures the upper trace shows the membrane potential and the lower trace is current monitor). A time-dependent depolarization is seen during the largest of the pulses. After termination of the pulse a delayed repolarization precedes the generation of spontaneous action potentials. *D*, a depolarizing current pulse evokes a series of action potentials and a post-pulse AHP is generated which causes a delay in the triggering of the succeeding spontaneous action potential. Note the spike-frequency adaptation during the pulse. *E*, superimposed records of the post-pulse AHPs generated after the termination of depolarizing pulses of various strength (pulse duration = 1·6 s, only late parts are shown). The amplitude and the duration of the AHPs are proportional to the strength of the preceding pulse. Records in *A–E* are from the same neurone.

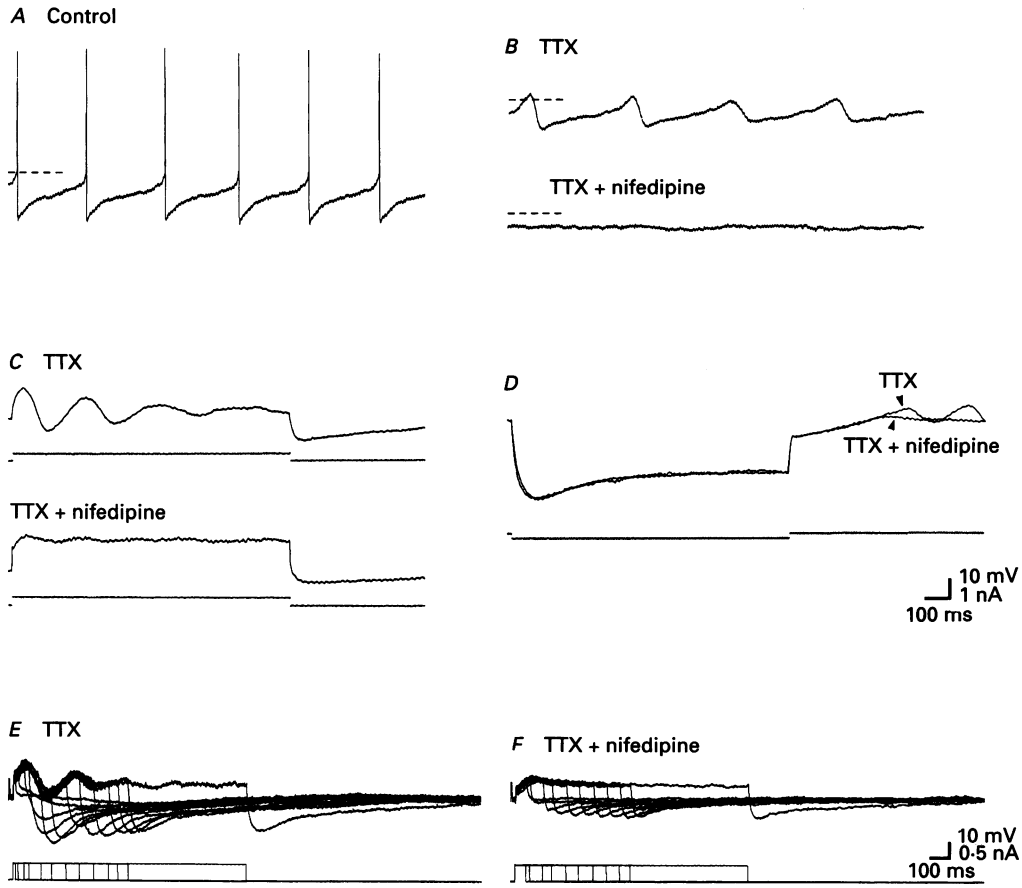

Fig. 2. Effect of nifedipine on the SOP in SNc neurones. *A* and *B*, spontaneous discharge of action potentials (*A*) is abolished when TTX is applied (*B*, upper trace) and the underlying SOPs are revealed. Note that their peak potential exceeds the firing threshold (−40 mV; indicated in each trace by a dotted line). After 25 min of perfusion with 0·5 μM nifedipine (*B*, lower trace) the spontaneous SOPs are completely abolished and the membrane potential is stabilized at −47 mV. *C* and *D*, in the presence of TTX a depolarizing current pulse evokes a series of SOPs (upper trace). After addition of nifedipine (0·5 μM, lower trace) a similar current pulse fails to evoke SOPs. The membrane input resistance was unchanged during nifedipine as revealed by superimposed traces of the voltage deflections in response to hyperpolarizing current pulses recorded before and during application of the drug (*D*). Records in *A–D* are from the same neurone. *E* and *F*, experiment from another neurone illustrating the influence of the SOP on the post-pulse AHP. Superimposed records of the voltage responses to depolarizing current pulses of varying duration show that the large AHPs following the termination of pulses display a pattern of fluctuation in amplitude which is correlated with the occurrence of SOPs (*E*). In the presence of 20 μM nifedipine (*F*) the SOPs are abolished and the post-pulse AHPs are of lower amplitude and show an increase in amplitude only related to the length of the preceding pulse.

accompanied by a gradual decrease in the amplitude and frequency of the spontaneous SOPs. After 15–25 min of application the SOPs were completely abolished (Fig. 2*B*). Furthermore, intracellular current injections, which had

previously facilitated the SOPs, were now incapable of generating the response (Fig. 2*C* and *D*). In some cells, however, a residual slow depolarization occurred at the initial part of a depolarizing pulse. This transient was of much lower amplitude than the SOP and did not involve a distinct hyperpolarizing phase. The blockade of the

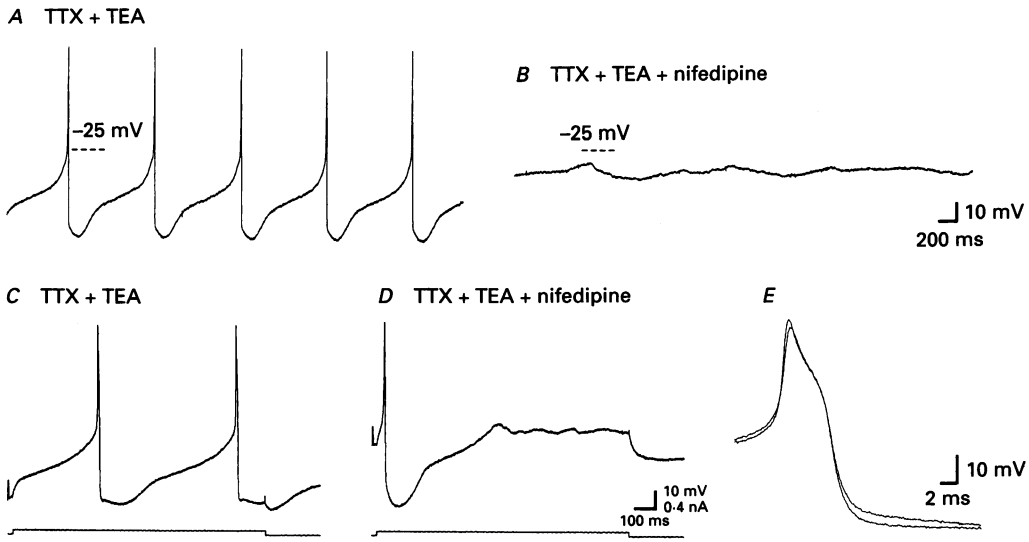

Fig. 3. Effects of nifedipine on the HTS in an SNc neurone. In the presence of TTX and 5 mM TEA, HTSs occur spontaneously at a regular frequency (*A*). Application of nifedipine (20 μM) abolishes the discharge of HTSs (*B*). HTSs can be evoked by injection of depolarizing current pulses (*C–E*); the waveform of a control HTS (*C*) is compared with one in the presence of nifedipine (*D*), and both are shown superimposed at an extended time scale (*E*). Note that the peak amplitude but not the duration of the HTS is slightly reduced by nifedipine. The AHP following the HTS is shorter in the presence of nifedipine (compare *C* and *D*).

SOP and the other effects of nifedipine, described below, showed no recovery even after 150 min wash-out of the drug. No difference was found between the effects of low (0·5–1 μM) and high (5–20 μM) concentrations of nifedipine, except in the delay of the response which was longer in low concentrations. Nifedipine application was not accompanied by any consistent alteration of the membrane input resistance; in sixteen out of twenty-two cells the input resistance was unchanged, a small decrease was observed in five cells, and a small increase in one cell.

During application of TTX alone a post-pulse AHP of considerable amplitude could be evoked, and hence this response did not entirely depend on the generation of spikes. Its absolute amplitude was found to vary with the duration of the preceding depolarization in a non-linear fashion. Indeed, it was correlated with the occurrence of SOPs, such that if the pulse was terminated near the peak depolarization of a SOP or during its falling phase an AHP with large amplitude was generated. Conversely, termination of a pulse during the rising phase of a SOP led to a comparatively smaller AHP (Fig. 2*E*). This phenomenon was not observed after the blockade of SOPs by application of nifedipine. Under such conditions the amplitude of the post-pulse AHP was markedly decreased and its size was merely dependent on the pulse duration (Fig. 2*F*).

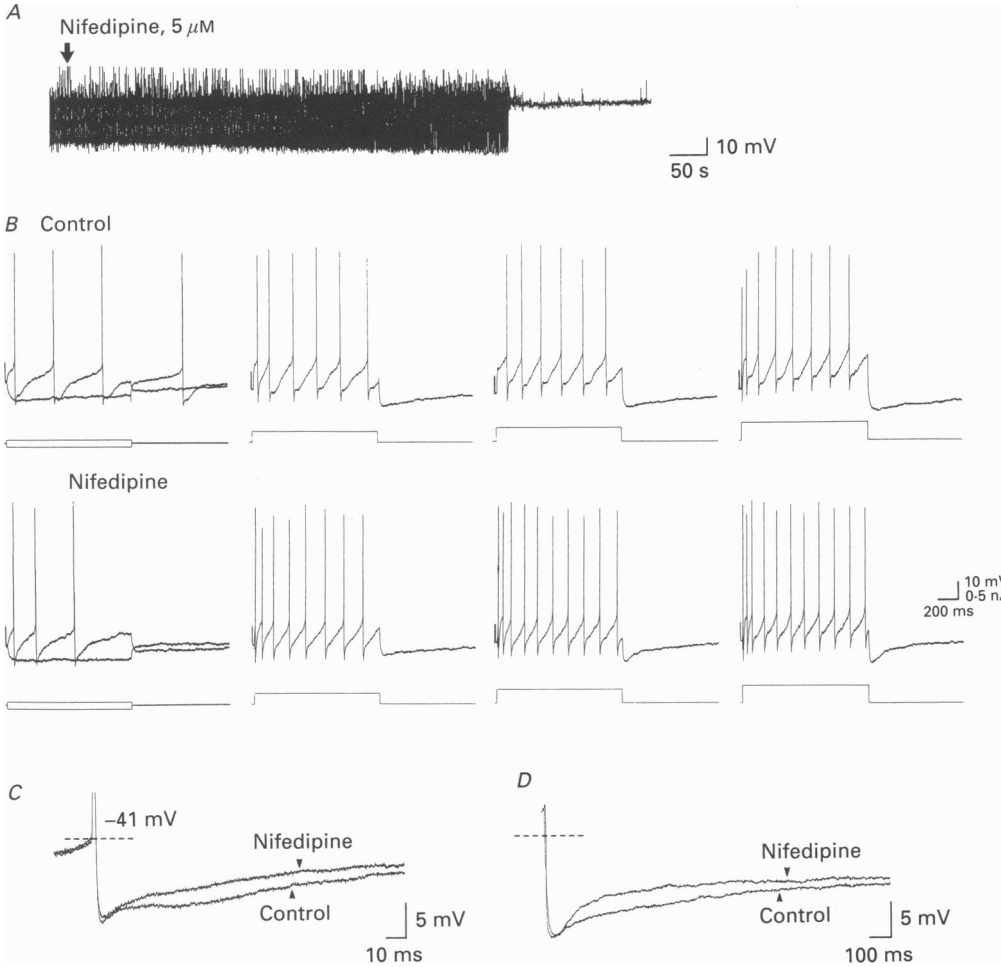

Fig. 4. Effects of nifedipine on the generation of action potentials and AHPs in SNc neurones. *A*, spontaneous discharge of action potentials (truncated) in normal medium is terminated after about 8 min of perfusion with 5 μM nifedipine (start indicated by arrow). The membrane potential stabilizes at −47 mV. *B*, the responses to injection of current pulses of varying polarity and strength are recorded in a control period (upper traces) and in the presence of 20 μM nifedipine (lower traces). There is no discernible effect of nifedipine on the membrane input resistance. However, although the spontaneous discharge is abolished, the frequency of action potentials generated by depolarizing current pulses is markedly enhanced by nifedipine. *C*, two superimposed records of single action potentials (truncated) evoked during a depolarizing pulse (0·1 nA; 1·1 s) from a control period and during application of 20 μM nifedipine. The two components of the spike AHP are discernible in the control record. During nifedipine application the late, slow component of the AHP is inhibited whereas the amplitude of the initial, fast component is only marginally reduced. *D*, two superimposed records of the AHP following a depolarizing current pulse (0·7 nA; 1·1 s; current trace not shown) from a control period and during nifedipine (20 μM). The maximal amplitude of the AHP (reached within the first 50 ms) is only slightly decreased by nifedipine. However, in the presence of nifedipine a markedly faster repolarization occurs during the following 150–200 ms, compared with the control. Dashed lines in *C* and *D* indicate the action potential threshold (−41 mV).

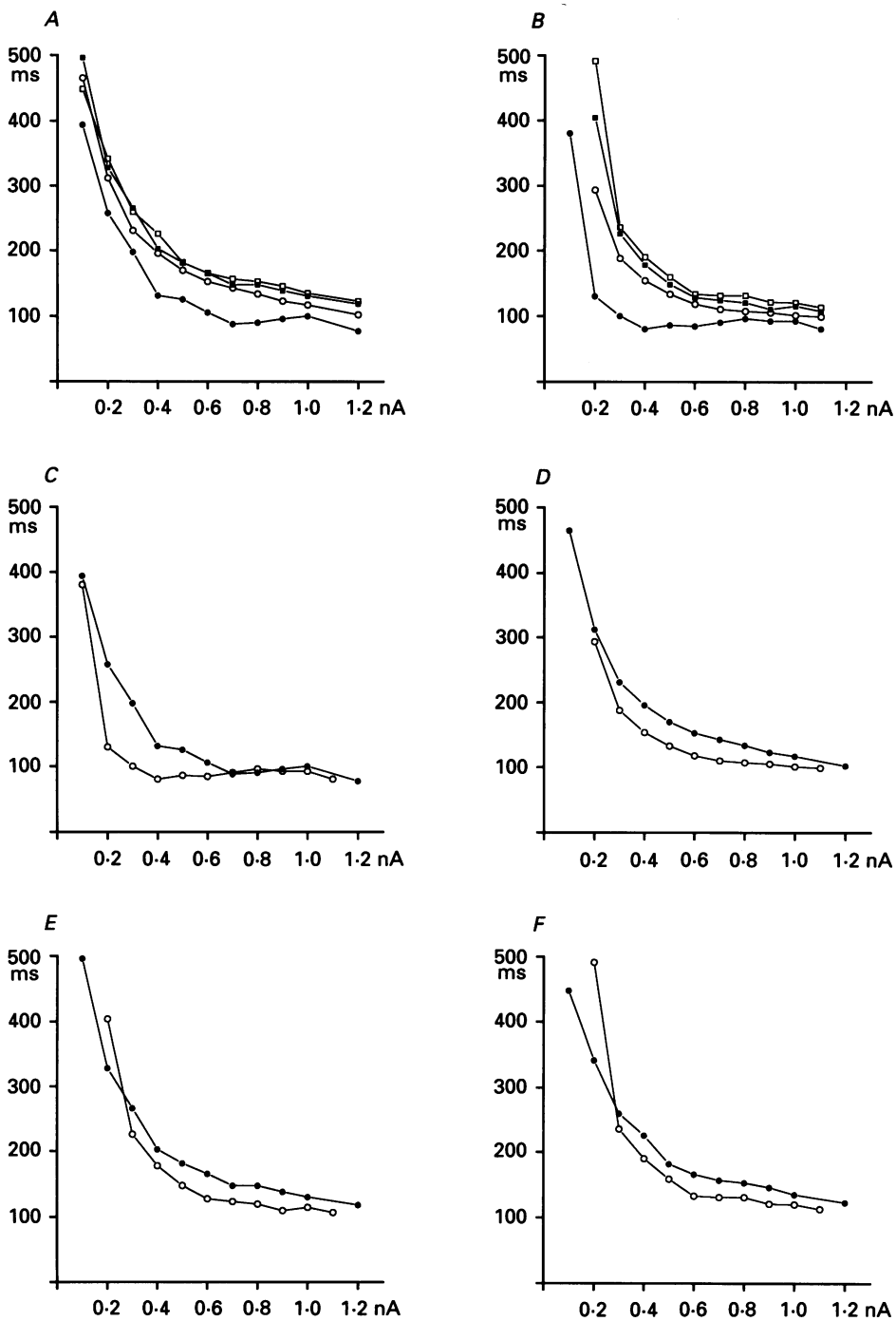

Fig. 5. Effects of nifedipine on the ISI in an SNc neurone. *A*, ISI–current plot (i.e. the ISI (*y*-axis) plotted against injected current (*x*-axis)) showing four curves which represent, respectively, the first ISI (●), and the ISI measured after 1 s (○), 2 s (■), and 3 s (□) of depolarization in a control period. Spike frequency adaptation is indicated by the increase in ISI with increasing pulse duration. The ISI–current relationship is non-linear; at lower current intensities the ISI is more affected by alteration in current than at larger

In the presence of K^+ channel blockers a second type of Ca^{2+}-mediated response can be isolated: the HTS (Kita *et al.* 1986; Grace & Onn, 1989; Nedergaard & Greenfield, 1992). The HTS is triggered at membrane potentials positive to −30 mV, it has a large amplitude and is followed by a marked, long-lasting AHP. In fifteen neurones the HTS was studied after blockade of the fast Na^+ spikes with TTX and with 5 mM TEA present in the perfusion medium. In ten cells the HTSs occurred spontaneously at a regular frequency. After addition of nifedipine (20 μM) the HTS discharge became irregular, and eventually it stopped completely (Fig. 3*A* and *B*). The spike was, however, not abolished by nifedipine, since it could still be evoked by injection of depolarizing current. The amplitude and duration of such evoked HTSs were found to be only marginally reduced in the present of nifedipine, as compared with the controls (Fig. 3*E*). In some of the neurones nifedipine application was accompanied by a marked reduction in the duration of the AHP following the HTS (Fig. 3*C* and *D*).

Since nifedipine thus seems to block selectively one of the two identified Ca^{2+}-dependent potentials in these neurones, valuable information may be obtained about their roles in the generation of the normal action potential and its components. Therefore, experiments were conducted in which nifedipine (1–20 μM) was applied during perfusion of otherwise normal medium. Out of eleven neurones tested, seven were spontaneously active prior to the application. in these cells the discharge of action potentials first became irregular and, after 7–15 min of application it was arrested (Fig. 4*A*). The membrane potential stabilized at $-50{\cdot}4 \pm 2{\cdot}9$ mV; $n = 7$. However, the frequency of action potentials generated by depolarizing current pulses was consistently *increased* in the presence of nifedipine (Fig. 4*B*). This increase in excitability was further investigated by plotting the interspike interval (ISI), measured at various times during the stimulation, against the strength of stimulation. During nifedipine application, a marked decrease was found in the ISI recorded over the whole range of a 3 s stimulation (Fig. 5). This effect was most pronounced during pulses of moderate strength (0·2–0·6 nA). The control ISI–current relationships showed a decreased slope with increasing current strength, and, in most of the neurones a 'minimal' ISI of 60–80 ms was attained. This was not altered by nifedipine. Furthermore, the spike frequency adaptation was still present during nifedipine application.

Comparison of single action potentials evoked in control periods and in the presence of nifedipine revealed a decrease in the duration of the AHP (Fig. 4*C*). This was due to a selective decrease in the late, slow component, since there was only little, if any, reduction in the peak amplitude of the AHP. Accordingly, the biphasic appearance of the spike AHP was less marked or, in some of the neurones, completely

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current intensities. *B*, same plots and symbols as in *A*, but data obtained in the presence of 20 μM nifedipine. The spike frequency adaptation is still evident. However, the non-linearity in the ISI–current relationship is now even more pronounced. This is further illustrated in *C–F* where corresponding data (as shown in *A* and *B*) from the control period (●) and in the presence of nifedipine (○) are shown in the same plot; the first ISI is shown in *C*, and the ISIs at 1, 2 and 3 s are shown in *D*, *E* and *F* respectively. There is an overall decrease in the ISI during nifedipine which is more pronounced at lower current intensities. Note that the first ISI (*C*) is not changed by nifedipine for current intensities larger than 0·6 nA.

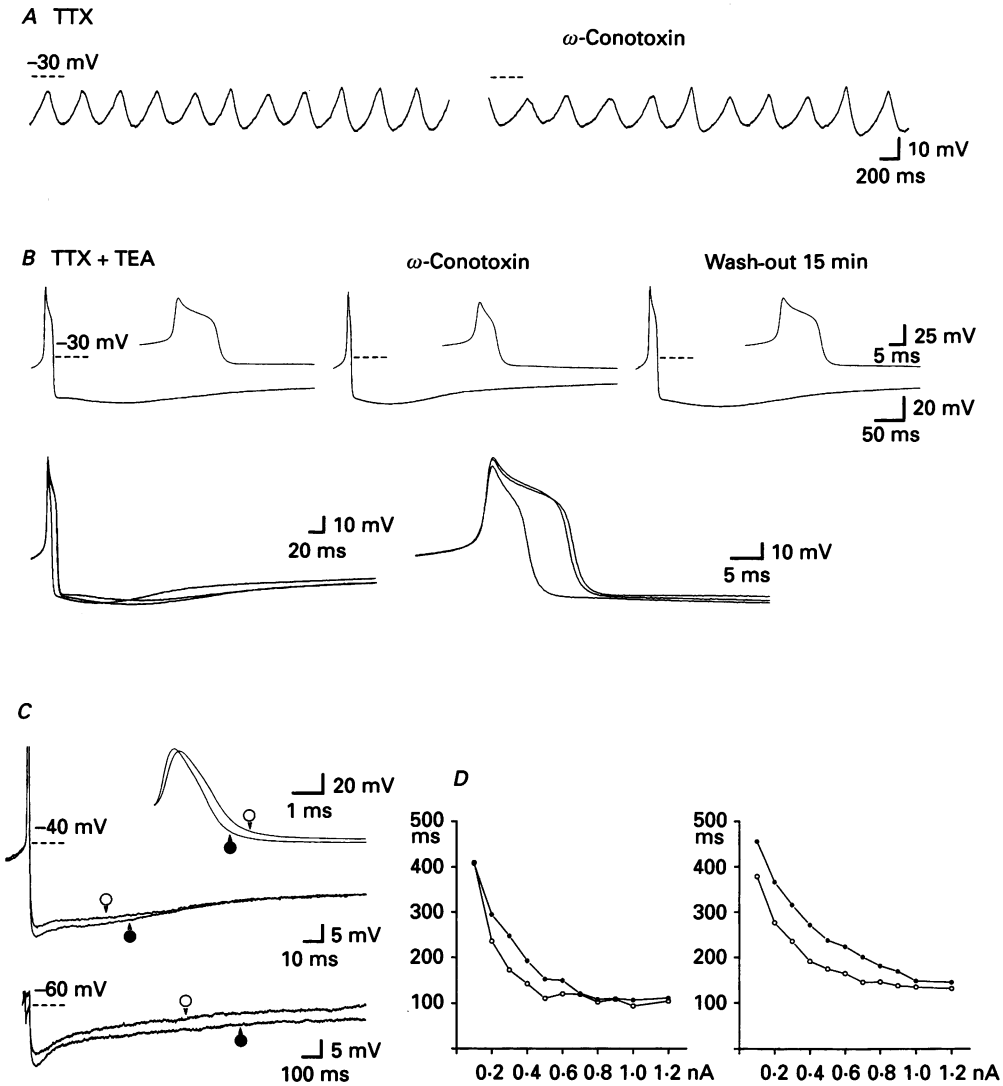

Fig. 6. Effects of ω-conotoxin on the Ca^{2+}-dependent potentials and the normal action potential in SNc neurones. *A*, in the presence of TTX spontaneous SOPs are generated at a regular rate (left). Application of ω-conotoxin (3 droplets) does not affect the amplitude or the frequency of the SOPs (right). *B*, in the upper traces records of spontaneously generated HTSs from a control period (left), during application of five droplets of ω-conotoxin (middle) and 15 min after wash-out of ω-conotoxin (right). Insets show the corresponding spikes at an extended time scale. All records are averages of four spikes. Dashed lines indicate −30 mV throughout. In the lower traces in *B* the three records are superimposed and shown at two different time scales. The duration and the amplitude of the HTS is decreased, and the duration of its AHP is reduced during application of ω-conotoxin. *C*, upper traces show superimposed records of single spontaneous action potentials (average of 4) from a control period in normal medium (●) and during application of ω-conotoxin (○). The fast, initial component of the spike AHP is clearly reduced by ω-conotoxin, whereas the slow component is still present. Inset shows the action potentials at their full heights. Note that the slopes of the rising and falling phases of the spike are decreased in the presence of ω-conotoxin. Lower traces in *C* show superimposed records of the AHP following depolarizing pulses (0·6 nA; 1·4 s; average of

abolished when nifedipine was present. The post-pulse AHP recorded in normal medium (or in the presence of TTX) was reduced substantially by nifedipine. In most neurones this response consisted of an isolated increase in the speed of repolarization during the first 200 ms of the AHP (Fig. 4*D*). The overall duration of the post-pulse AHP was not decreased by nifedipine.

No consistent alteration in the waveform of the SOP was observed during application of either octanol (100–200 μM; $n = 3$), amiloride (250 μM; $n = 4$), or low concentrations of Ni^{2+} (100–300 μM, $n = 4$). The SOP was attenuated when Ni^{2+} was applied in 500 μM concentration ($n = 5$).

Inhibition of the high threshold Ca^{2+} spike by ω-conotoxin

The effects of the peptide ω-conotoxin were recorded in thirteen neurones. Application of this drug was performed either via the perfusion solution (0·5–10 μM), or by droplets (containing 50 μM ω-conotoxin in saline) delivered to the surface of the slice. In the presence of TTX neither the spontaneous nor the evoked SOPs were significantly altered in shape by ω-conotoxin (Fig. 6*A*). However, the HTSs appearing after the addition of 5 mM TEA were markedly inhibited in the presence of ω-conotoxin (Fig. 6*B*). Typically, this response involved a decrease in the duration of the spike, whereas the amplitude was less affected. In two cells, however, both the height and the duration of the HTS were reduced substantially (not illustrated). In all the neurones tested, the duration of the AHP following the HTS was reduced during application of ω-conotoxin. Droplet applications were more efficacious than bath applications; thus evoked, the responses occurred rapidly (< 1 min) and showed full recovery over a period of 15–20 min after the application (Fig. 6*B*). The effects could also be induced repeatedly in the same cell without showing desensitization. Conversely, the effects induced during bath applications were irreversible throughout the recordings (i.e. up to 90 min wash-out).

The effects of ω-conotoxin were also monitored in six cells during perfusion with normal medium. No noticeable effect of the drug was observed in frequency or pattern of the spontaneous discharge, and the membrane input resistance was unaltered. In three neurones a decrease in the ISIs recorded during depolarizing current injections was found (Fig. 6*D*). The other three neurones showed no significant change in the ISI–current relationship during ω-conotoxin application. However, the AHP following a single action potential and the post-pulse AHP were consistently reduced by ω-conotoxin (Fig. 6*C*). The effect on the single-spike AHP was clearly different from that observed during nifedipine application since it involved a distinct reduction of the fast component. It was not possible, from the records, to determine if the slow component of the AHP was also inhibited. If so, this effect was not as marked as that produced by nifedipine, since a biphasic waveform of the AHP was still discernible in all the neurones during ω-conotoxin application. Figure 6*C* shows that ω-conotoxin reduced the slopes of the rising and the falling phases of the action potential. This response occurred normally without a

2). The amplitude of the AHP is decreased during ω-conotoxin application. Symbols as in upper traces. *D*, ISI–current plots from a SNc neurone (axes as in Fig. 5). This cell responded to ω-conotoxin (○) by a decrease in the ISI as compared with the control (●). Left, first ISI; right, the ISI measured after 1 s of stimulation. Note the lack of effect of ω-conotoxin on the first ISI recorded at current strengths larger than 0·6 nA.

concomitant reduction in the spike amplitude. No effect of ω-conotoxin was found on the shape of Na^+ action potentials recorded in Ca^{2+}-free, Co^{2+}-containing medium ($n = 4$).

Effects of apamin

Some of the effects described above are likely to result from alterations in a K^+ conductance activated by Ca^{2+} influx. Therefore, these effects were compared with

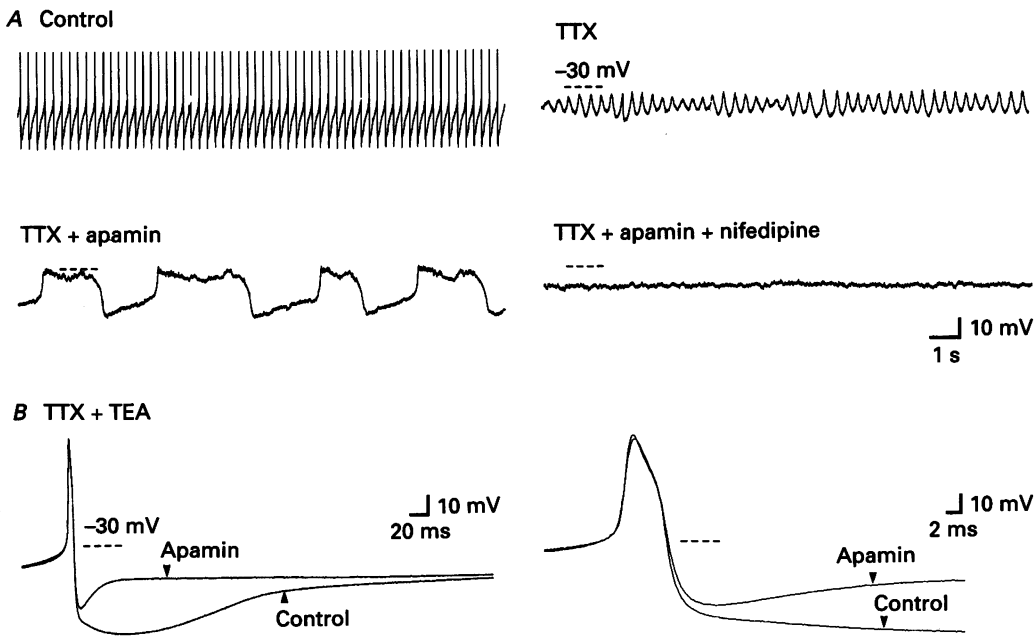

Fig. 7. Effects of apamin on the Ca^{2+}-dependent potentials in SNc neurones. *A*, spontaneous discharge of action potentials (truncated) in normal medium (upper left) is blocked by TTX leaving the SOPs (upper right). Addition of apamin (0·1 μM) for 15 min induces depolarizing voltage plateaux, which occur spontaneously (lower left). Each plateau is terminated by a large, relatively fast hyperpolarization which is immediately followed by a slow, ramp-like depolarization. At a threshold of approximately −45 mV a relatively fast depolarization results in the next plateau. When nifedipine (20 μM) is subsequently added the plateaux are completely abolished (lower right). Dashed lines indicate −30 mV in all traces. *B*, superimposed records of spontaneously generated HTSs (average of 4) from a control period and during application of apamin (0·1 μM). The spike AHP is markedly inhibited in both amplitude and duration by apamin (left). The HTSs are shown at an extended time scale to the right. Note that the height and duration of the HTS is unchanged during apamin application.

those induced during application of apamin, a known antagonist of Ca^{2+}-activated K^+ channels (Blatz & Magleby, 1986). The responses to bath applications of apamin (0·05–0·2 μM) were tested in a total of eleven neurones. In the presence of TTX, apamin induced a gradual slowing of the spontaneously occurring SOPs. The effect was maximal after approximately 10 min, at which time the rhythmic oscillations were replaced by long-lasting plateau-like potentials (Fig. 7*A*). Thus, the membrane potential shifted spontaneously between a relatively stable level around −35 mV, and a level 25–30 mV more negative. The latter state was characterized by a slow,

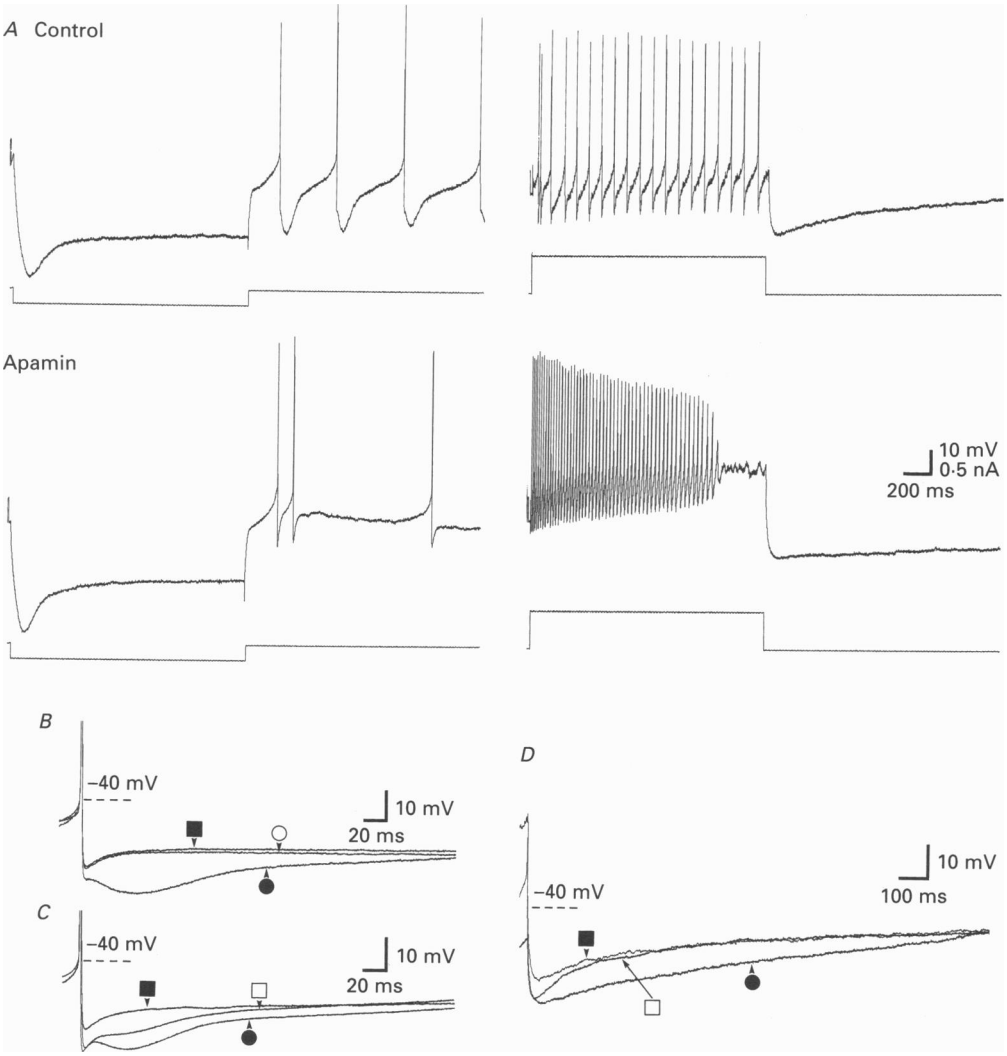

Fig. 8. Effects of apamin on the action potential generation and AHPs in SNc neurones. *A*, the responses to membrane polarization by current pulses are shown from a control period (upper traces) and during application of 0·1 μM apamin (lower traces). A hyperpolarizing pulse (left) reveals a slightly increased input resistance during apamin; termination of the pulse is followed by a slow rebound depolarization which cannot be seen in the control record. During apamin application a depolarizing pulse generates spikes at a much higher frequency than during the control period. Note the spike frequency adaptation and the progressive decrease in spike amplitude during the pulse. Spike inactivation occurs at the end of the pulse. *B*, action potentials from the same cell as shown in *A* are superimposed. Records are from a control period (●), during application of 0·1 μM apamin (○), and during application of both apamin and 20 μM nifedipine (■). Note the decrease in both the fast and slow component of the AHP by apamin. Addition of nifedipine has only little further effect on the waveform of the spike AHP. *C*, superimposed action potentials from another cell where nifedipine (20 μM) was applied before apamin (0·1 μM) was added. Nifedipine (□) selectively reduces the slow component of the spike AHP, whereas apamin (■) causes a further reduction in the slow component and also inhibits the fast, initial component of the AHP. ●, control. Each trace is an average of ten spikes. *D*, superimposed records of AHPs following termination

ramp-like depolarization which resulted in the triggering of the next plateau at about −45 mV. Injections of short polarizing current pulses could trigger the potential shift in either direction. These voltage excursions were completely abolished when nifedipine (20 μM) was added to the medium (Fig. 7*A*).

In the presence of TTX and TEA apamin induced a dramatic decrease in the duration of the AHP following the HTS (Fig. 7*B*). The peak amplitude of the AHP was also reduced, but a fast component of considerable size persisted in the presence of apamin. There were no discernible changes in the shape of the HTS itself.

Applications of apamin during recordings in normal medium, resulted in the following responses: (i) a shift in the spontaneous firing pattern from regular to irregular discharge (the average spike frequency was either unchanged or slightly increased), (ii) an increase in membrane input resistance, and (iii) a marked enhancement of the membrane excitability. The latter effect is illustrated in Fig. 8*A*. During apamin application depolarizing current pulses evoked a burst of action potentials with progressively decreasing amplitude and frequency, which proceeded to complete spike inactivation. Burst firing could also be evoked as a rebound response following the termination of hyperpolarizing current pulses. In this case spikes were generated on a slow depolarization (Figs 8*A* and 9*A*), which was not visible in normal medium. Addition of nifedipine (20 μM) did not block the apamin-induced ability to generate bursts (Fig. 9*A*).

In all the neurones tested apamin abolished the slow component of the AHP following single action potentials (Fig. 8*B*). It caused a minor, but definite, reduction in the fast component. Addition of nifedipine under such conditions did not further reduce the AHP. Conversely, in experiments where nifedipine was added prior to the apamin application, the selective inhibition of the slow component of the AHP produced by nifedipine was found to be further accentuated by apamin (Fig. 8*C*). Furthermore, apamin was more efficacious than nifedipine in reducing the post-pulse AHP. Thus, nifedipine preferentially induced a fast repolarization during the initial period of the AHP, with little effect on its amplitude. Apamin, on the other hand, consistently reduced the amplitude of the post-pulse AHP (Fig. 8*D*). The overall duration of the post-pulse AHP was not altered by apamin when pulses generating an equal number of spikes were compared. All the effects of apamin described above showed no recovery during wash-out periods of up to 90 min.

Identification of TTX-sensitive plateau potentials

In order to examine whether a Ca^{2+}-independent mechanism may be involved in the nifedipine-resistant generation of bursts, observed during apamin application (Fig. 9*A*), the effects of removal of Ca^{2+} were studied in thirteen neurones. Substitution of Ca^{2+} with Co^{2+} in the perfusion solution caused an almost complete abolishment of both components of the spike AHP, and the slope of the spike repolarization was decreased (see Nedergaard & Greenfield, 1992). Under these circumstances, injection of depolarizing current pulses was found to generate series of sustained depolarizations (Fig. 9*B*). Na^+ spikes occurred on top of these voltage

of depolarizing current pulses (1·6 s; 1·0 nA). Application of 50 μM nifedipine (□) enhances the repolarization of the AHP without reducing its amplitude, as compared with the control (●). Addition of 0·1 μM apamin (■) causes a marked inhibition of the AHP amplitude. Each trace represents an average of four records.

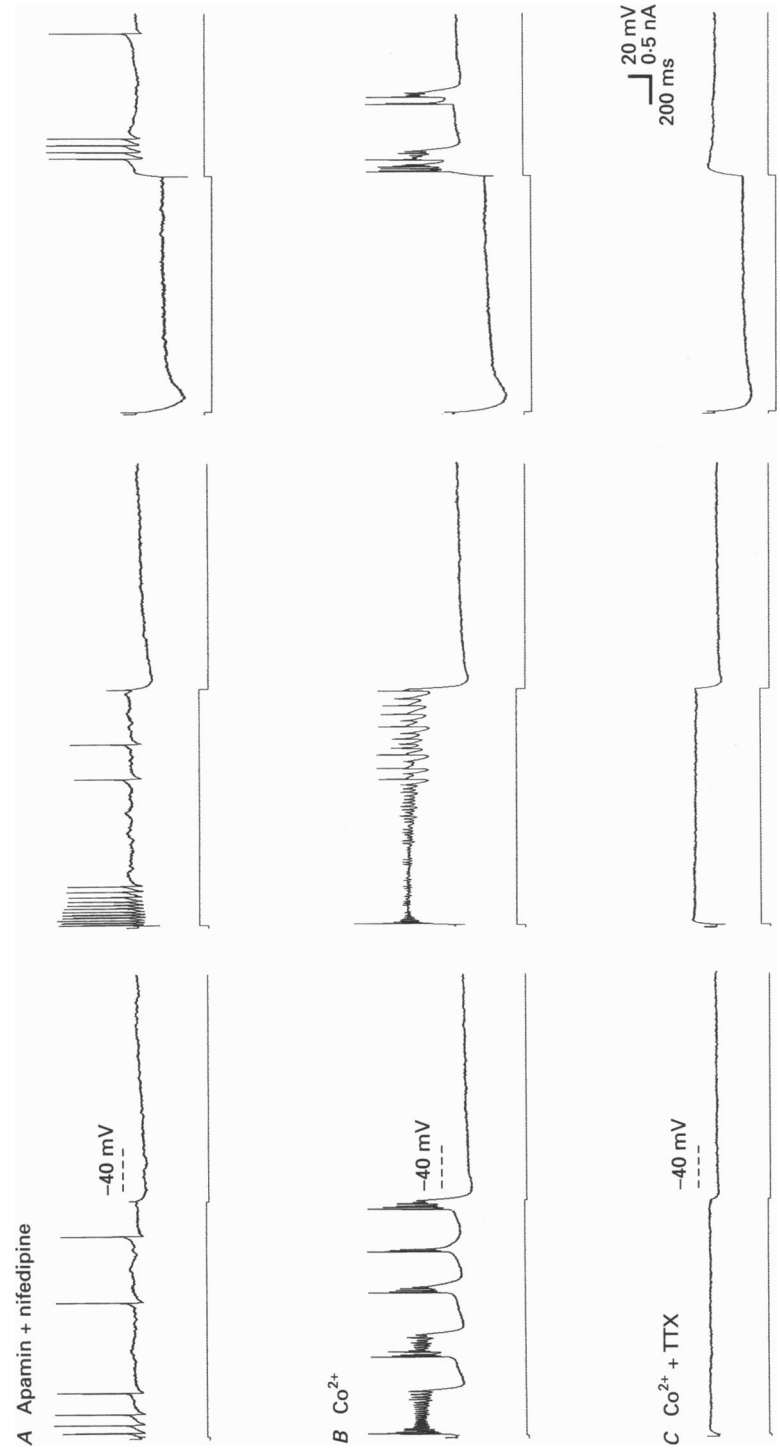

Fig. 9. TTX-sensitive voltage plateaux in a SNc neurone. *A*, in the presence of apamin (0·1 μM) and nifedipine (20 μM) depolarizing current pulses (left and middle) evoke a burst of action potentials. A burst is also generated after the termination of a hyperpolarizing pulse (right). *B*, after 40 min perfusion with Ca^{2+}-free Co^{2+}-containing medium a depolarizing pulse evokes action potentials, which ride on a series of depolarizing voltage plateaux (left). A stronger current pulse generates a sustained voltage plateau (middle). Plateaux are also evoked as rebounds following termination of a hyperpolarizing pulse (right). *C*, repetition of the pulse protocol shown in *A* and *B*, but after addition of TTX to the perfusion solution. Both the action potentials and the plateaux are abolished.

plateaux at high frequencies (although spike inactivation occurred rapidly during prolonged depolarization). The duration of the responses was proportional to the strength of the injected current, and, in some cells one or more rebound plateaux were generated after termination of a hyperpolarizing current pulse (Fig. 9*B*). The voltage plateaux could be evoked irrespective of whether apamin was present in the medium. Addition of TTX completely abolished both the Na^+ spikes and the voltage plateaux (Fig. 9*C*).

DISCUSSION

Calcium channel subtypes underlying the SOP and the HTS

The results presented in this study show that at least two different types of regenerative Ca^{2+}-mediated responses can be distinguished in the SNc neurones, and the each of these make specific contributions to the membrane response properties. The SOP, which has previously been identified by several investigators of these neurones (Fujimura & Matsuda, 1989; Harris *et al.* 1989; Grace, 1991; Yung *et al.* 1991) is shown here to be eliminated in the presence of nifedipine. This finding suggests that the SOP is mediated via L-type Ca^{2+} channels, which are potently inhibited by dihydropyridines (Fox, Nowycky & Tsien, 1987; Aosaki & Kasai, 1989; Plummer, Logothetis & Hess, 1989). In isolated neurones it has been found that high concentrations of dihydropyridines (10 μM) may have non-specific effects on N-type Ca^{2+} currents (Jones & Jacobs, 1990; Regan, Sah & Bean, 1991) and cause reduction in Na^+ and K^+ currents (Jones & Jacobs, 1990). Such effects cannot be excluded entirely. However, we found that 0·5 μM nifedipine was sufficient to block the SOP (in a brain slice this may correspond to an even lower concentration in the vicinity of the neurone) which suggests that this effect is selectively on the L-type channels. The SOP occurs at membrane potentials negative to the HTS threshold. This is consistent with findings in other cells that dihydropyridine-sensitive Ca^{2+} currents activate at more hyperpolarized potentials than the ω-conotoxin-sensitive currents (Regan *et al.* 1991; Kasai & Neher, 1992).

In our experiments the SOP was not blocked by the known T-channel antagonists octanol, amiloride or low concentrations of Ni^{2+} (see Fox *et al.* 1987; Llinás, 1988; Crunelli, Lightowler & Pollard, 1989; Mogul & Fox, 1991). However, application of Ni^{2+} in concentrations higher than 300 μM did attenuate the SOP, which agrees with the findings of Yung *et al.* (1991). Whether this latter effect is due to a selective blockade of T-channels or, alternatively, reflects a non-specific inhibition of Ca^{2+} channels remains to be shown. A minor contribution from T-channels in the generation of the SOP cannot be excluded here. It should also be considered that currents other than those mediated by Ca^{2+} influence these oscillations. Thus, a slow TTX-sensitive potential has been shown to participate in the depolarization preceding action potentials (Grace & Onn, 1989; and see below). Also the I_h (slow, mixed inward rectifier current), which, in the SNc neurones, activates at around -50 mV (Nedergaard, Flatman & Engberg, 1991), and the outward rectifier current, are likely to contribute to the waveform of the SOP.

While nifedipine had only a marginal inhibitory effect on the HTS, this latter response proved to be sensitive to ω-conotoxin. A complete blockade of the HTS was, however, not accomplished even with high concentration (10 μM). Thus, it is possible

that the HTS is not mediated via a single type of Ca^{2+} channel. Furthermore, the ω-conotoxin response was reversible after local applications (the slow onset and lack of reversibility during bath applications may be due to slow diffusion of the drug within the tissue). This is in contrast with other studies where the blockade of N-type Ca^{2+} channels by ω-conotoxin is found to be irreversible (Kasai, Aosaki & Fukuda, 1987; McCleskey *et al.* 1987). In one study, however, recovery of ω-conotoxin effects has been reported which occurs within a time frame comparable to that observed here (Morrill, Boland & Bean, 1991). It has also been suggested that ω-conotoxin causes a transient blockade of a fraction of the dihydropyridine-sensitive (L-type) current (Aosaki & Kasai, 1989) or the T-current (McCleskey *et al.* 1987), and Plummer *et al.* (1989) found two ω-conotoxin-sensitive components of the N-type current, one that was blocked irreversibly by ω-conotoxin and one that was blocked reversibly. Because of these discrepancies it seems difficult from the present data to establish the identity of the HTS currents, and particularly the role of the N-type channels. However, ω-conotoxin had no detectable effects on the SOP. This, together with the poor sensitivity of the HTS to nifedipine, gives reason to assume that the two voltage transients are mediated via different types of Ca^{2+} channels.

Role of the SOP in spike generation and in control of excitability

Blockade of the rhythmic discharge of action potentials has previously been demonstrated in SNc neurones during perfusion with inorganic calcium antagonists (Fujimura & Matsuda, 1989; Grace, 1990; Nedergaard & Greenfield, 1992). In the present study it was found that nifedipine consistently abolished the spontaneous firing. This strongly suggests that the SOP is necessary for the triggering of action potentials during spontaneous discharge. The results also indicate that the Ca^{2+} influx occurring during SOPs contributes significantly in the generation of a K^+ conductance responsible for the slow component of the spike AHP. The nifedipine-sensitive component of the AHP was found to be effectively abolished by apamin. Such correspondence in action between nifedipine and apamin was further substantiated by the finding that apamin transformed the SOP into a voltage plateau. This phenomenon is most simply explained by a selective blockade, by apamin, of the hyperpolarizing phases necessary for recycling of the SOP. Plateaux were not seen in the absence of TTX; however, in a recent study where apamin was applied to SNc neurones in higher concentrations (1 μM), similar waveforms were induced in normal medium and were found to underlie bursting activity (Shepard & Bunney, 1991). These authors conclude that the slow, apamin-sensitive component of the AHP plays a role in regulating the steady firing pattern and the membrane excitability, but not in spike-frequency adaptation. The present study confirms this interpretation, since the indirect blockade of the slow AHP by nifedipine induced irregular firing (before the complete blockade of discharge) and a general increase in excitability. Nifedipine did not seem to interfere with the spike-frequency adaptation or the total duration of the post-pulse AHP. Such properties correspond closely to a slow Ca^{2+}-dependent K^+ conductance underlying the medium-duration AHP (mAHP) described in cortical neurones (Schwindt, Spain, Foehring, Stafstrom, Chubb & Crill, 1988). The early part of the post-pulse AHP probably reflects cumulative activation of single spike AHPs since it was sensitive to nifedipine and apamin. However, it is noteworthy that the post-pulse AHP is not entirely

dependent on the firing of action potentials. Indeed, sustained activation of the SOP can, by itself, generate a large post-pulse AHP. The physiological significance of the latter phenomenon seems unclear, since, under normal circumstances, the fast K^+ conductances may serve to inactivate the SOP by a sufficient hyperpolarization between each spike.

Previous studies suggest that only slow Ca^{2+}-dependent K^+ conductances are sensitive to apamin (Lazdunski, 1983; Pennefather, Lancaster, Adams & Nicoll, 1985; Kawai & Watanabe, 1986; Zhang & Krnjević, 1987). The apamin-induced decrease in the fast component of the spike AHP suggests that the slow conductance is already active in the early AHP (Sánchez & Ribas, 1991). This may indicate that the onset of this conductance is very fast compared to its decay. However, an alternative explanation is provided by the experiment illustrated in Fig. 2*E*, where the SOP-induced AHP facilitation is shown to be induced before the SOP reaches its peak depolarization. Hence, the slow Ca^{2+}-dependent K^+ channels may open before the triggering of the action potential and therefore, contribute to the early AHP.

Role of the HTS in the spike repolarization and in the fast component of the AHP

The responses observed during application of ω-conotoxin in normal medium indicate that the currents underlying the HTS (i.e. the Ca^{2+} current, I_{Ca} and the Ca^{2+}-activated K^+ current, $I_{K(Ca)}$) participate in the repolarization of the normal action potential and in the generation of the fast component of the AHP. The slow AHP component may also be generated by this mechanism, as indicated by the reduced amplitude of the post-pulse AHP (see above), and the decreased ISIs recorded during ω-conotoxin application. Qualitatively similar alterations in the action potential and the AHP are induced by the removal of Ca^{2+} from the external medium (Yung *et al.* 1991; Nedergaard & Greenfield, 1992). It is therefore likely that the effects of ω-conotoxin on the action potential are due to the blockade of the HTS current, especially since it did not seem to affect the fast Na^+ conductance.

A large proportion of the fast AHP following both the HTS and the normal action potential was found here to be insensitive to apamin. Hence, at least two pharmacologically distinct Ca^{2+}-dependent K^+ conductances are involved in the AHP complex. Since the fast component is not generated as a part of the SOP, it can be inferred that Ca^{2+} influx *per se* is not sufficient to activate it. It is likely, therefore, that the underlying K^+ conductance is both Ca^{2+} and voltage dependent, i.e. it activates only at membrane potentials which are positive to the firing threshold. This is in line with the finding that it is the K^+ currents with a fast decay time that are distinguished by a requirement for both increased intracellular Ca^{2+} concentration and depolarization for activation (Brown, Constanti & Adams, 1983). In addition, the fast AHP component is sensitive to TEA (Grace, 1990), and hence its properties seem to correspond to those of the voltage-dependent, Ca^{2+}-activated K^+ current, I_c (Brown & Griffith, 1983; Pennefather *et al.* 1985; Lancaster & Adams, 1986).

Slow sodium-dependent voltage transients in SNc neurones

In Ca^{2+}-free, Co^{2+}-containing medium, voltage-dependent plateau potentials were observed to underlie burst discharge of fast spikes. These TTX-sensitive plateaux indicate that a persistent Na^+ conductance exists in the SNc neurones. It may

explain why neurones which were exposed to both apamin and nifedipine displayed a slow depolarizing waveform which was found to underlie bursting. Since the SOPs were eliminated, these latter depolarizations may be generated by the persistent Na^+ conductance. It is possible that this conductance is normally activated together with the slow Ca^{2+} conductance and thus participate in the SOP.

The present data suggest that the slow rhythmic discharge of the SNc neurones depends critically on the cyclic activation of L-type Ca^{2+} channels. The opening of these channels is responsible for triggering of action potentials via the initiation of the SOP. The waveform of the SOP is, in turn, the result of an interplay among a variety of Ca^{2+}-, Na^+- and K^+-dependent conductances. In order to understand further how this complex interaction can integrate into a highly monotonous discharge pattern, more studies are needed to establish the properties and the subcellular localization of the channels involved.

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