Dopamine D_1 receptor modulates the voltage-gated sodium current in rat striatal neurones through a protein kinase A

Serge N. Schiffmann*, Pierre-Marie Lledo and Jean-Didier Vincent

Institut Alfred Fessard, CNRS, 91198 Gif-sur-Yvette, France

1. Whole-cell recordings were made from striatal neurones obtained from neonatal rats and maintained in primary cultures. The effects of dopamine D_1 receptor activation were studied on the voltage-gated sodium current.

2. Bath application of a specific D_1 agonist, SKF38393 (1 μM), reduced the neuronal excitability recorded in current-clamp by increasing the threshold for generation of action potentials.

3. In voltage-clamp recordings, SKF38393 (1 μM) reversibly reduced the peak amplitude of the sodium current by $37{\cdot}8 \pm 4{\cdot}95\%$. This effect was reversed by the D_1 antagonist SCH23390 and was blocked by the intracellular loading of GDP-β-S (2 mM) suggesting GTP-binding protein involvement.

4. The D_1 agonist reduced the peak amplitude of the sodium current without significantly affecting (i) the voltage dependence of the current–voltage relationship, (ii) the voltage dependence of the steady-state activation and inactivation, (iii) the kinetics of the time-dependent inactivation, and (iv) the kinetics of recovery from inactivation.

5. The peak amplitude of the sodium current was progressively reduced by intracellular loading of cyclic AMP-dependent protein kinase (100 U ml^{-1}).

6. Diffusion of a specific peptide inhibitor of the cyclic AMP-dependent protein kinase (PKI; 10 μM) into the cytosol of neurones blocked the effect of the D_1 agonist on the sodium current amplitude.

7. These results demonstrate that dopamine acting at the D_1 receptor reduces the amplitude of the sodium current without modifying its voltage- and time-dependent properties. This effect involves activation of the cyclic AMP-dependent protein kinase and results in a depression of the striatal neuronal excitability by increasing the threshold for generation of action potentials.

Striatal neurones are one of the main targets for dopamine in the central nervous system. Since they are implicated in the control of motor functions and are involved in many motor dysfunctions (for recent reviews see Albin, Young & Penney, 1989; Gerfen, 1992; Strange, 1993), it is of crucial importance to understand how dopamine affects their electrophysiological properties.

Dopamine exerts pleiotropic effects which reflect both the existence of multiple dopamine receptors and the coupling of a single receptor to different effectors by multiple transducing pathways (for reviews, see Sibley & Monsma, 1992; Strange, 1993). The dopamine receptors have been first subclassified into two categories: the D_1 and D_2 receptors characterized by their ability to stimulate and inhibit, respectively, the production of cyclic AMP by adenylate cyclase in striatal neurones (Stoof & Kebabian, 1981). Cloning of genes encoding these receptors revealed the existence of other subtypes which have been classified into two subgroups: D_1 and D_5 in the D_1 receptor subfamily and D_2, D_3 and D_4 in the D_2 receptor subfamily (for reviews see Sibley & Monsma, 1992; O'Dowd, 1993). The striatum expresses mostly the D_1 and D_2 receptors (Gerfen, 1992; Strange, 1993), and to a lesser extent the D_3 subtype (Sibley & Monsma, 1992; O'Dowd, 1993; Strange, 1993). In the present paper, we shall use the terms D_1 and D_2 receptors in the broad application including all dopamine receptor subtypes.

* To whom correspondence should be addressed at: Brain Research Unit and Laboratory of Neuropathology and Neuropeptides Research, Université Libre de Bruxelles, Campus Erasme, CP 601, 808 route de Lennik, 1070 Brussels, Belgium.

The modulation of voltage-gated potassium and calcium channels by stimulation of the dopamine D_2 receptor has been extensively studied in endocrine tissues such as the anterior pituitary gland (Stack & Surprenant, 1991; Lledo, Homburger, Bockaert & Vincent, 1992), and the identification of the α-subunit of the heterotrimeric GTP-binding protein (G protein) involved in the transduction pathway has been previously reported (Kleuss, Hescheler, Ewel, Rosenthal, Schultz & Wittig, 1991; Lledo *et al.* 1992). On the other hand, while the dopamine-mediated regulation of cyclic AMP levels and the transduction mechanism(s) leading to this regulation have been extensively characterized in striatal neurones (Stoof & Kebabian, 1981; Sibley & Monsma, 1992; Strange, 1993), the effects of dopamine on the membrane properties, and therefore on the control of the excitability of these neurones, are less understood. In line with this, the transduction mechanism(s) resulting from dopamine receptor(s) activation are still unknown. Freedman & Weight (1988) reported the opening of a non-voltage-gated potassium channel after D_2 receptor activation and Surmeier and colleagues (Surmeier, Eberwine, Wilson, Cao, Stefani & Kitai, 1992) described a modulation of the voltage-gated sodium channel activity resulting from the activation of at least the D_1 and the D_2 receptors.

The modulation of ionic channel activity by neurotransmitters and hormones, either through a direct G protein coupling or through the production of second messengers has been extensively documented, especially for calcium and potassium currents, in many excitable tissues (for reviews see Nicoll, Malenka & Kauer, 1990; Catterall, 1992). Because voltage-gated sodium channels are responsible for the initiation and propagation of the action potential (Eccles, 1964), modulation of their activity is expected to affect dramatically neuronal excitability. In different cellular preparations, activation of the β-adrenergic (Schubert, Vandongen, Kirsch & Brown, 1989), thyrotropin-releasing hormone (Lopez-Barneo, Castellano & Toledo-Aral, 1990), dopamine (Surmeier *et al.* 1992) and FMRFamide receptors (Brussaard, Lodder, Ter Maat, de Vlieger & Kits, 1991) have been reported to modulate a voltage-gated sodium current. However, the transduction mechanisms involved in all of these neurotransmitter effects remains largely unknown. Regulation could either involve a direct coupling of an α-subunit of G proteins, or a covalent modification through phosphorylation by cyclic AMP-dependent protein kinase (PKA) or calcium-dependent protein kinase (PKC). Recently, modulation of a sodium current by arachidonic acid has also been reported in striatal neurones (Fraser, Hoehn, Weiss & MacVicar, 1993) suggesting a novel mechanism by which neuronal excitability can also be modulated.

In the present report, we investigated the biophysical and molecular mechanisms of the sodium current modulation by the dopamine D_1 receptor on striatal neurones maintained in primary cultures. The effects of a specific D_1 agonist were assessed on the activation and inactivation properties of the sodium current and the putative involvement of PKA was tested by using intraneuronal dialysis of the catalytic subunit of PKA or of a specific inhibitor of this kinase (PKI). Our data demonstrated that activation of the dopamine D_1 receptor depressed the amplitude of the sodium current through the activation of the PKA, leading to a reduction of neuronal excitability by increasing the threshold for generation of action potentials. Preliminary and partial accounts of this work have appeared in abstract form (Schiffmann, Lledo & Vincent, 1994).

METHODS

Primary culture of striatal neurones

Four- to five-day-old Wistar pups (P4–P5) (Iffacredo, Saint Germain, France) were aseptically decapitated and placed in phosphate-buffered saline (PBS) containing 33 mM D-glucose. Following the removal of the brains from the crania, the dorsal striata were dissected in cold PBS–glucose. The minced striata were pooled and treated for 1 min with 250 μg ml^{-1} trypsin (Gibco BRL, Eragny, France) at 37 °C. Following centrifugation at 200 *g* and two rinses, they were treated with 15 μg ml^{-1} of DNAse I diluted in serum-free medium. The minced striata suspension was gently triturated using fire-polished Pasteur pipettes and the resulting suspension was centrifuged (3 × 5 min at 200 *g*) and rinsed in culture medium to remove cellular debris. Cells were then plated onto 35 mm diameter Petri dishes at a density ranging between 0·8 and 1×10^6 cells per dish. Petri dishes had been previously coated with 15 μg ml^{-1} poly-ornithine, rinsed with sterile water and thereafter coated with 3 μg ml^{-1} laminin. The culture medium consisted of Eagle's minimal essential medium supplemented with sodium bicarbonate (2·2 g l^{-1}), L-glutamine (0·73 g l^{-1}), glucose (3·6 g l^{-1}), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), 10% horse serum and cytosine arabinosine (2 μM) to prevent non-neuronal proliferation. Cultures were maintained in a humid, 5% CO_2 atmosphere at 37 °C and half of the medium was changed once a week. Culture medium and sera were obtained from Gibco; all other salts and drugs were purchased from the Sigma Chemical Company (St Louis, MO, USA).

Whole-cell voltage-clamp recordings of the sodium current

Striatal neurones (12–21 days *in vitro*) were recorded using the tight-seal whole-cell mode of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with a high-gain voltage-clamp amplifier (RK 300 Biologic, Claix, France). For recordings, the Petri dish containing the cultured neurones was fixed on the stage of an inverted Nikon Diaphot microscope equipped with Nomarsky optics (Nikon, Champigny-sur-Marne, France). Patch pipettes were fabricated from borosilicate capillary tubing (1·5 mm o.d., GC150TF-10, Clark Electrical Instruments, Reading, UK) and pulled on a PP-83 micropipette puller (Narishige Scientific Instruments, Tokyo, Japan). They presented resistances of 4–8 MΩ when filled with the patch pipette solution (see below). Junction potential between the electrode solution and the bath was adjusted to zero and membrane potential values were not corrected with

regard to this liquid junction potential. Series resistances with cell capacitances were compensated using the procedure described in the RK 300 manual.

Membrane currents were filtered using an eight-pole Bessel low-pass filter (10 kHz, 3 dB, Frequency Devices, Haverhill, MA, USA) and all current traces (each current trace was an average of three consecutive records elicited at 0·3 Hz) had capacitance and leakage currents subtracted digitally from hyperpolarizing command pulses using a patch-clamp analysis program, VCAN, provided by Dr J. Dempster (University of Strathclyde, Glasgow, UK).

The bath solution was continuously perfused using gravity flow at a rate of 0·5–1 ml min^{-1} (bath solution volume around 1 ml). Patch-clamp experiments were conducted at room temperature (21–24 °C). To isolate the sodium current, the extracellular recording solution contained (mM): 50 NaCl, 100 tetraethylammonium chloride (TEA-Cl), 1 $MgCl_2$, 1 $CaCl_2$, 1 $CoCl_2$, 5 $CsCl_2$, 10 D-glucose and 10 Hepes, adjusted to pH 7·3 and 300–330 mosmol l^{-1}. The pipette solution contained (mM): 65 di(Tris)phosphate, 40 Tris-base, 5 CsCl, 11 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 0·4 Na_3GTP, 4 Na_2ATP, 0·2 cyclic AMP, 20 phosphocreatine, 0·1 leupeptin, 10 D-glucose, 10 Hepes and 50 U ml^{-1} creatine phosphokinase, adjusted to pH 7·3 and 270–290 mosmol l^{-1}. In order to reach the gigaseal cell-attached configuration, the tip of the pipette was back-filled with this intracellular solution without creatine phosphokinase, leupeptin, phosphocreatine or any other protein or peptide (see below) in all experiments.

Whole-cell current-clamp recordings

For current-clamp recordings, the intracellular solution contained (mM): 135 potassium gluconate, 11 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 0·4 Na_3GTP, 4 Na_2ATP, 0·2 cyclic AMP, 20 phosphocreatine, 0·1 leupeptin, 10 D-glucose, 10 Hepes and 50 U ml^{-1} creatine phosphokinase, adjusted to pH 7·3 and 270–290 mosmol l^{-1}. Cells were bathed in external solution containing (mM): 140 NaCl, 5 $CaCl_2$, 1 $MgCl_2$, 4 KCl, 10 D-glucose and 10 Hepes, adjusted to pH 7·3 and 300–330 mosmol l^{-1}.

Drugs

Depending upon experimental protocols, these basic solutions were modified by appropriate addition of compounds and drugs. The catalytic subunit of PKA (Promega Corporation, Madison, WI, USA) at 100 U ml^{-1}, 10 μM of the PKA inhibitor 5-24 (PKI 5-24) (Peninsula Inc., Belmont, CA, USA) or 2 mM GDP-β-S (Sigma Chemical Co.) were added to the intracellular solution. Stock solutions of SKF38393 (RBI, Natick, MA, USA), SCH23390 (RBI), ketanserin (Sigma Chemical Co.) dissolved in dimethyl sulphoxide at 10 mM and tetrodotoxin (Sigma Chemical Co.) dissolved in distilled water at 1 mM were added to the bath solution to give adequate final concentrations. Bath solutions were exchanged using gravity flow.

RESULTS

Morphology of striatal neurones

Striatal neurones (12–21 days *in vitro*) were used for electrophysiological experiments. The majority of the neurones, examined 1–3 weeks after plating, typically had a diameter of 5–10 μm (Fig. 1*A*), an ovoid and symmetric soma with two primary neurites or an ovoid and asymmetric somatic shape with multiple primary neurites. These two subgroups of neurones most probably correspond to the IA and IB classes of striatal neurones in primary cultures characterized by their morphological and immunohistochemical properties (Surmeier, Kita & Kitai, 1988), despite the fact that they have been prepared from postnatal animals (P4–P5) instead of embryos (E17). They represent more than 80% of neurones and a large proportion of them are immunoreactive for γ-aminobutyric acid (Surmeier *et al.* 1988). Although this has not been definitely demonstrated, Surmeier *et al.* (1988) inferred from their data that these IA and IB neurones correspond in the adult striatum to the medium-sized spiny neurones which comprise 80–90% of the neuronal population and express dopamine receptors such as D_1 or D_2 subtypes (Gerfen, 1992). Since the type IA and IB neurones in our cultures are expected to belong mostly to the medium-sized spiny neurone subpopulation and therefore to be dopaminoceptive, we chose to restrict our recordings to them. Although we cannot exclude that some of our recorded neurones belong to another neuronal type such as the medium-sized aspiny interneurones, the very low proportion of the latter as compared with that of the medium-sized spiny neurones render this putative error insignificant.

Electrophysiological properties

In all experiments and protocols, neurones with relatively short processes were chosen for recordings in order to optimize the space control of voltage-clamp conditions. The decay phase of the capacitive transient was well described by a single exponential and the time constant for activation of the sodium current was fast and clearly showed the same voltage dependency as previously reported (see Fig. 1*B* and Ogata & Tatebayashi, 1990; Fraser *et al.* 1993). Moreover, the voltage-dependent activation of the sodium current was gradual (see superimposed current traces in Fig. 1*B* and the current–voltage relationship in Fig. 3*B*) and the tail current was well fitted by a single exponential (data not shown). Altogether this argued for adequate voltage-clamp conditions.

The electrophysiological properties of these neurones were first investigated in the current-clamp mode.

The mean resting membrane potential value corresponding to the zero-current potential was $-42 \pm 2{\cdot}1$ mV (mean ± S.E.M.; $n = 14$) when measured immediately on achieving whole-cell mode, matching previous results (cf. Freedman & Weight, 1988; Fraser *et al.* 1993). For sixteen neurones tested in voltage-clamp mode, the mean input resistance was $3{\cdot}1 \pm 0{\cdot}4$ GΩ (mean ± S.E.M.) at membrane potentials close to the resting membrane potential. Striatal neurones never exhibit a spontaneous firing, although low-frequency firing could be elicited with injection of either a modest tonic depolarizing current or depolarizing current pulses which allowed membrane potentials to reach the threshold for generation of sodium-dependent action

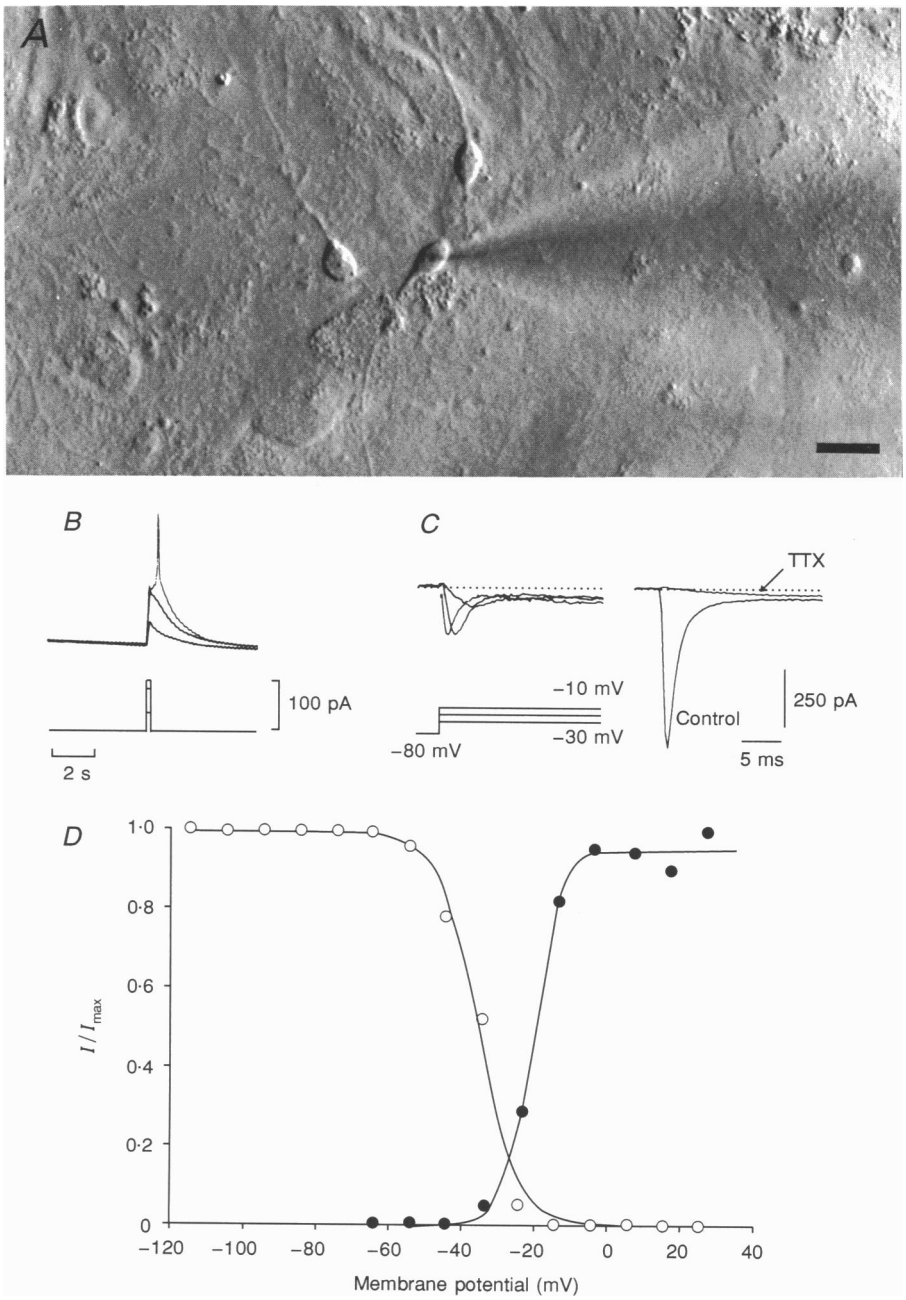

Figure 1. Morphology and basic electrophysiological properties of the striatal neurones in primary cultures

A, striatal neurones in primary culture representative of the neurones recorded for this study. These neurones had a diameter of 7–9 μm, exhibited an ovoid and symmetric somatic shape and had two primary neurites. Scale bar, 20 μm. *B*, search for the threshold for generation of a typical action potential by injection of depolarizing currents of increased amplitude in current-clamp mode and recorded from a holding potential of −80 mV. *C*, in voltage-clamp and in conditions allowing the isolation of sodium currents, depolarizing voltage steps to indicated potentials from a holding potential of −80 mV evoked a fast and transient inward current which activated approximately at −40 mV, peaked at −10 mV and was TTX sensitive. *D*, representative steady-state activation and inactivation were obtained from peak currents and were fitted by Boltzmann equations:

$$\text{Inactivation: } I/I_{max} = 1/\{1 + \exp[(V - V_h)/k]\},$$

where V is the conditioning potential, V_h is the potential at mid-point and k the slope factor for the inactivation curve, and:

$$\text{Activation: } G/G_{max} = 1/\{1 + \exp[(V - V_h)/k]\},$$

where V is the test potential, V_h is the potential at mid-point, k the slope factor, and $G = I/(V - E_{rev})$ where E_{rev} is the extrapolated reversal potential from the current–voltage curve.

potentials (Fig. 1*B*). The mean threshold was −30·6 ± 1·3 mV and the amplitude of the action potential was 37·2 ± 2·6 mV (mean ± S.E.M.; $n = 16$).

Voltage-gated calcium, potassium and, to a lesser extent, sodium currents expressed by striatal neurones in primary cultures or acutely dissociated have been extensively characterized (Surmeier, Bargas & Kitai, 1989; Ogata & Tatebayashi, 1990; Surmeier, Stefani, Foehring & Kitai, 1991; Fraser *et al.* 1993; Hoehn, Watson & MacVicar, 1993). Therefore the sodium current was first briefly characterized in control conditions using the voltage-clamp mode. In the whole-cell recording configuration the initial inward currents were analysed after suppressing the outward currents by internal Tris and abolishing the calcium currents by external cobalt ions. When the membrane potential was depolarized, these neurones developed a fast and transient inward current (Fig. 1*C*). This inward current first appeared at a potential around −40 mV (ranging from −45 to −35 mV) and peaked between −25 and −10 mV, as demonstrated by superimposed current traces evoked by test pulses from a holding potential of −80 mV (Fig. 1*C*).

Representative steady-state activation and inactivation are illustrated in Fig. 1*D* and exhibit all characteristics previously described for a sodium current (Ogata & Tatebayashi, 1990; Surmeier *et al.* 1992; Fraser *et al.* 1993). Since, in addition, this current persisted in the presence of 1 mM cobalt and was completely blocked by 1 μM tetrodotoxin (Fig. 1*C*), it was concluded that the inward current is carried through the voltage-gated sodium channels.

Effects of the D_1 agonist SKF38393 on action potentials

The effect of a specific agonist of the dopamine D_1 receptor, SKF38393, at a final concentration of 1 μM was first examined in the current-clamp mode. As shown in Fig. 2, the bath application of SKF38393 abolished the action potential generated by the injection of a depolarizing current previously adjusted to reach the threshold for generation of the action potential ($n = 6$). A subsequent increase of the injected current, in the presence of the agonist, allowed a new threshold to be obtained and therefore again elicited an action potential similar to the previous one (Fig. 2) or slightly reduced in amplitude (data not shown). It is noteworthy that the application of 1 μM SKF38393 did not modify consistently the resting membrane potential; this agonist either slightly hyperpolarized, depolarized or did not change its value (see Fig. 2).

Modulation of the sodium current by the D_1 agonist SKF38393

Since the D_1 agonist increases the threshold for generation of action potentials, one possibility to explain such an effect includes a D_1 receptor modulation of the voltage-gated sodium current. We have therefore investigated the effect of SKF38393 during voltage-clamp experiments. Figure 3*A* shows that the application of the D_1 agonist depressed the amplitude of the sodium current evoked by a 15 ms test pulse to −20 mV, from a holding potential of −80 mV (a mean reduction of 37·8 ± 4·9%; $n = 22$). This effect was reported for 64% of the recorded neurones and was completely reversible upon wash-out. Moreover, the D_1-induced effect was not subject to desensitization since a second application of 1 μM SKF38393 after recovery again depressed the amplitude of the sodium current to a similar extent (Fig. 3*A*). Similarly, the response did not desensitize during a longer exposure.

This decrease in sodium current amplitude was partially reversed by the subsequent bath application of a specific D_1 receptor antagonist, SCH23390 (3–10 μM; $n = 5$; Fig. 4*A*),

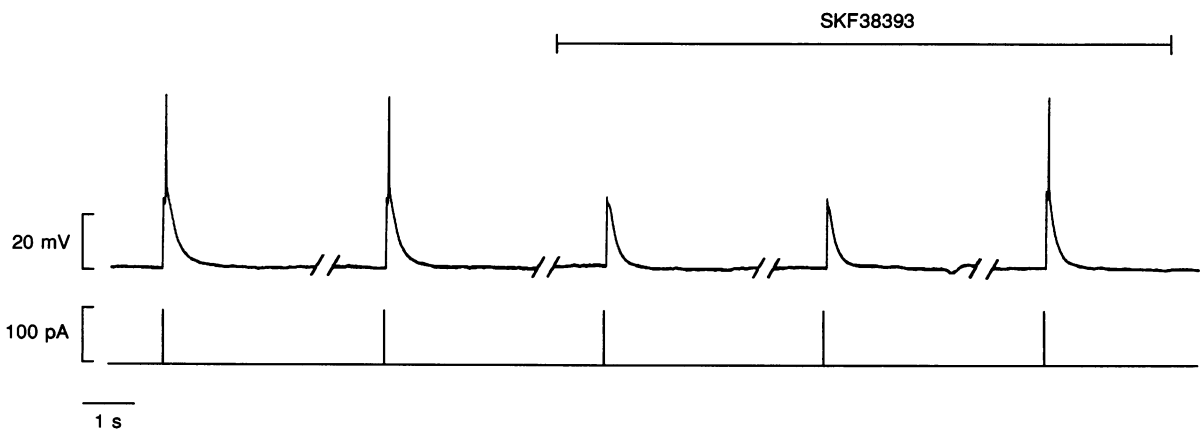

Figure 2. Effect of the dopamine D_1 agonist SKF38393 (1 μM) on elicited action potentials recorded under current-clamp mode

The injection of a 10 ms suprathreshold depolarizing current from a potential of −70 mV elicited a typical action potential which is abolished by the application of SKF38393. A subsequent increase of the injected current allows a new threshold for generation of an action potential to be reached (right trace).

the precentages of recovery ranging from 70 to 100%. Conversely, SKF38393, which could bind with a low affinity to the 5-HT_2 receptor (Woodward, Panicker & Miledi, 1992), still reduced the amplitude of the sodium current in the presence of the 5-HT_2 receptor antagonist ketanserin (1 μM), by $27 \pm 4{\cdot}3\%$ in 60% of five tested neurones (Fig. 4*B*). These results demonstrated that SKF38393 affected the amplitude of the sodium current through a specific activation of the dopamine D_1 receptor.

When neurones were loaded with guanosine 5′-*O*-(2-thiodiphosphate) (GDP-β-S), a non-hydrolysable analogue of GDP which prevents activation of G proteins, the depression of the sodium current amplitude induced by SKF38393, 6–8 min after reaching the whole-cell recording configuration, was abolished in all six neurones tested (data not shown). This result demonstrated therefore that the effect of the D_1 agonist is transducted through the activation of a GTP-binding protein.

Effects of the D_1 agonist on steady-state gating properties of the Na^+ current

Current–voltage relationship

The current–voltage relationship was obtained by applying 15 ms depolarizing pulses at a frequency of 0·3 Hz, from a holding potential of −80 mV and the current amplitudes were measured at the peak inward current. The current–voltage relationships obtained before and during treatment with 1 μM SKF38393 (Fig. 3*B*) showed a marked decrease in the magnitude of the sodium current through the entire range at which the cell membrane potential was stepped. Moreover, the potential level at which the sodium current became maximal in either the presence or absence of SKF38393 was

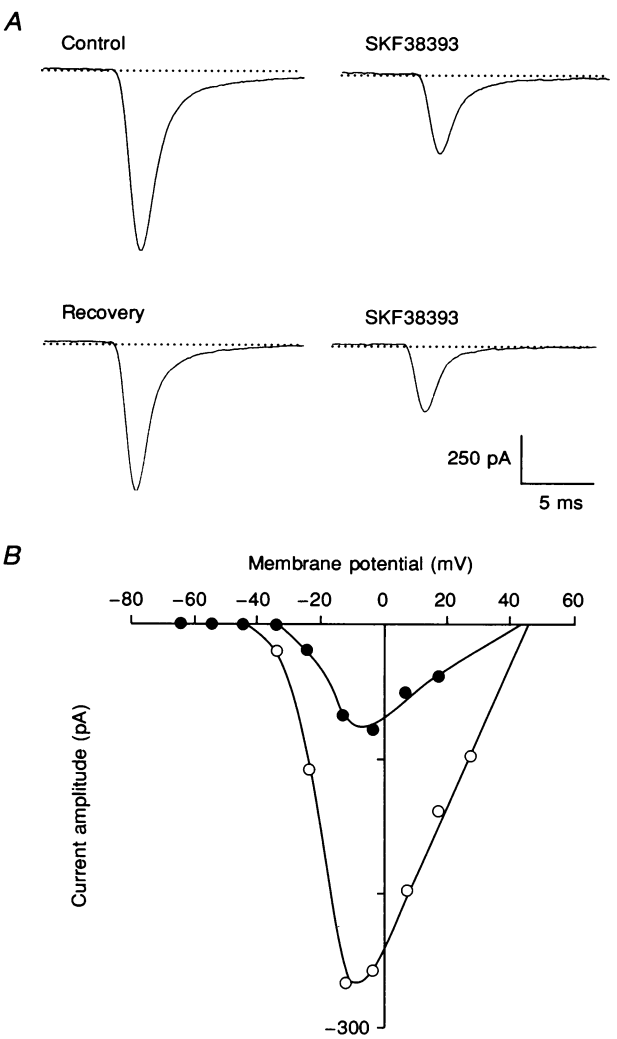

Figure 3. Activation of D_1 receptors induces reduction of voltage-gated sodium current

A, SKF38393 reversibly depressed the peak amplitude of the sodium current in a non-desensitizing manner. In this and following similar figures, 15 ms test pulses to −20 mV were applied from a holding potential of −80 mV. *B*, current–voltage relationships demonstrate the non-voltage-dependent depression in sodium current after application of the D_1 agonist (○, control; ●, SKF38393).

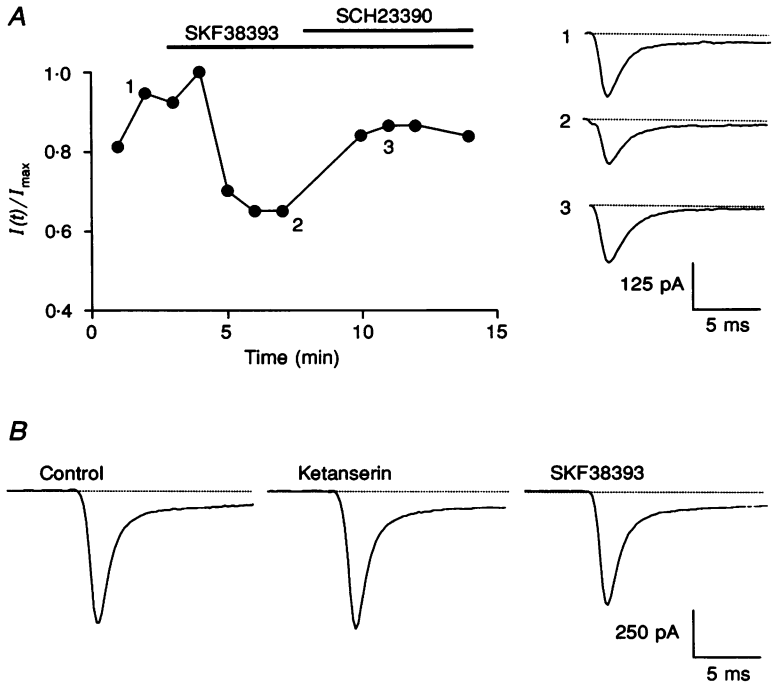

Figure 4. The effect of SKF38393 was specifically mediated by the dopamine D_1 receptor

A, the inhibitory effect of SKF38393 was blocked by a specific D_1 receptor antagonist, SCH23390. Bars indicate the application of SKF38393 and SCH23390, respectively. Current traces 1–3 shown in inset were recorded at the times indicated in the graph. *B*, the 5-HT_2 receptor antagonist, ketanserin, neither affects the sodium current by itself (middle trace) nor abolishes the effect of SKF38393 (right trace).

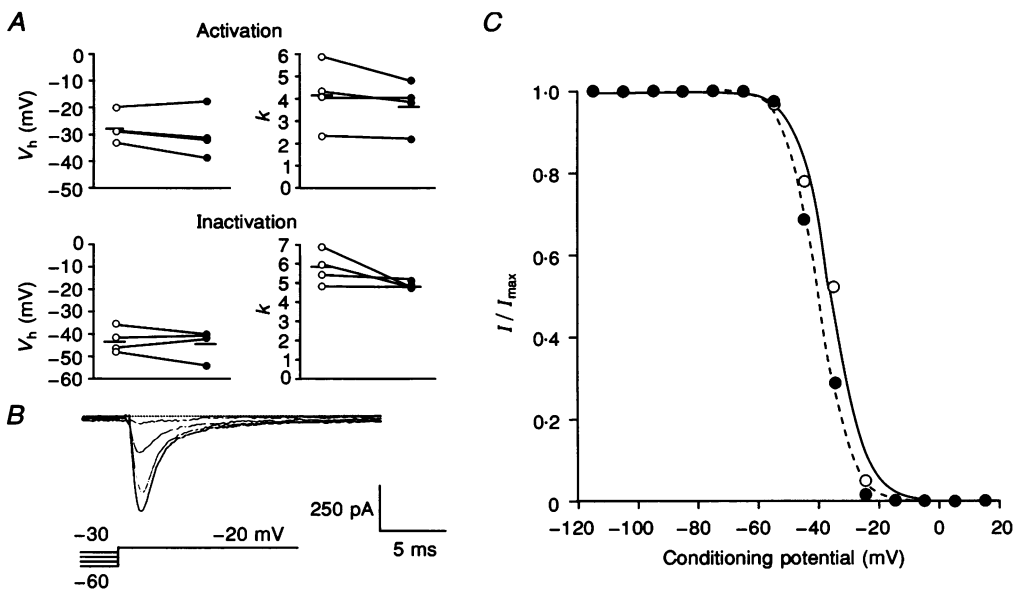

Figure 5. Absence of effects of SKF38393 on activation and inactivation properties

A, scatter plots of V_h (potential at mid-point) and k (slope factor) values for individual neurones show that SKF38393 did not significantly affect either the steady-state activation or inactivation (○, control; ●, SKF38393). The steady-state activation and inactivation curves were fitted by the Boltzmann equations $G/G_{max} = 1/\{1 + \exp[(V_h - V)/k]\}$ and $I/I_{max} = 1/\{1 + \exp[(V_h - V)/k]\}$, respectively (see text and legend of Fig. 1 for more details). *B*, representative current traces obtained in control conditions using the illustrated two-pulse protocol for study of steady-state inactivation. *C*, steady-state inactivation curves for a representative neurone before and after application of SKF38393 (○, control; ●, SKF38393).

unchanged, and the decrease was maximal at such potential levels ($n = 7$). Changes in sodium current threshold or in the extrapolated reversal potential were never seen. Therefore it appeared that the main effect of D_1 receptor activation was to decrease the maximal conductance of the sodium current.

Activation relationship

The steady-state activation relationship was constructed from the current–voltage curves. The activation curve was estimated by calculation of $G = I/(V - E_{rev})$ where E_{rev} is the extrapolated reversal potential from the current–voltage curve and G, the macroscopic conductance. The calculated conductances were normalized, plotted as a function of the test potential and the curves were fitted by the Boltzmann equation:

$$G/G_{max} = 1/\{1 + \exp[(V_h - V)/k]\},$$

where V is the test potential, V_h is the voltage at which half the current is activated and k, the slope factor, represents the voltage dependence of the activation process. The application of 1 μM SKF38393 did not significantly modify the voltage-dependent steady-state activation measured in four tested neurones since, before and after application of the D_1 agonist, values for V_h were $-27{\cdot}8 \pm 2{\cdot}8$ and $-29{\cdot}7 \pm 4{\cdot}4$ mV (mean ± S.E.M.) and for k were $4{\cdot}2 \pm 0{\cdot}7$ and $3{\cdot}7 \pm 0{\cdot}5$, respectively (Fig. 5*A*).

Inactivation relationship

The effect of D_1 agonist was also investigated on the voltage-dependent steady-state inactivation, using a two-pulse protocol. The potential was held for 30 ms, successively from −120 to +20 mV before application of a constant test pulse to −20 mV (Fig. 5*B*). The steady-state inactivation curve was determined by normalizing the peak amplitude of the sodium current during the test pulse

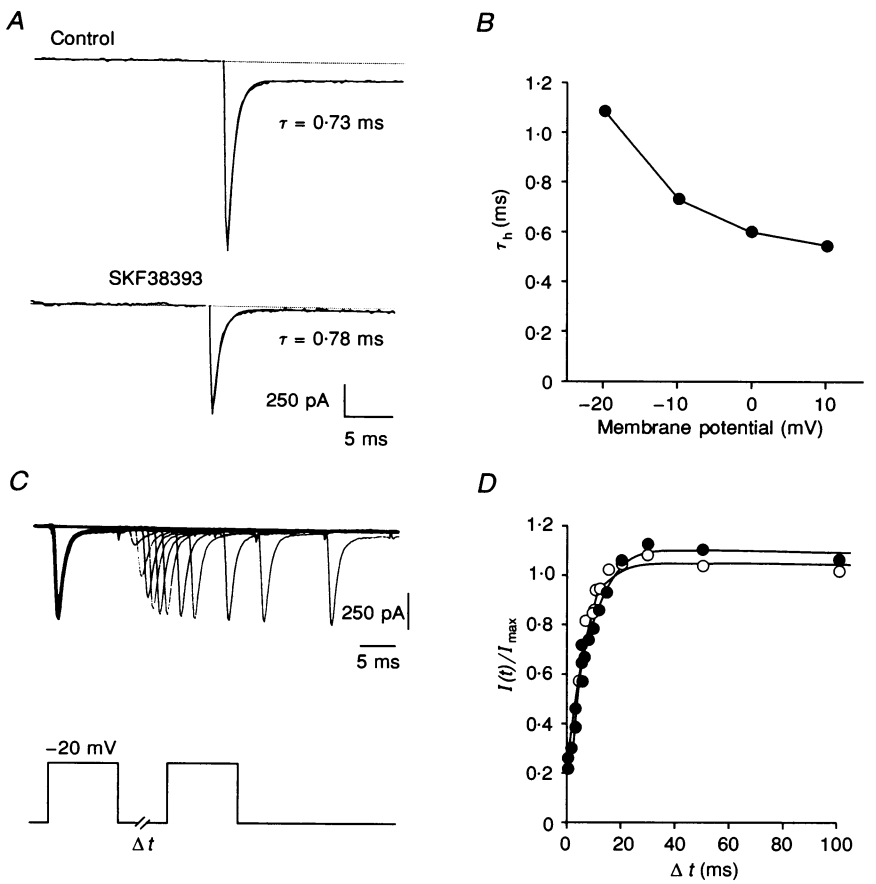

Figure 6. Absence of effects of SKF38393 on inactivation kinetics

A, representative fits to inactivation of sodium currents at −10 mV in control and SKF38393 conditions. *B*, τ_h as a function of the test membrane potential for a representative neurone in control conditions. *C*, representative superimposed current traces illustrating the kinetics of recovery from inactivation in control conditions (with an interpulse duration ranging from 0 to 30 ms). *D*, relationship between the normalized current and the interpulse duration for a representative neurone demonstrating the absence of effect of SKF38393 (○, control; ●, SKF38393). For this particular neurone, $\Delta t_{½}$ for recovery from inactivation was 4·5 ms before and after application of SKF38393.

as a function of the conditioning potential (Fig. 5*C*). These curves were fitted by the Boltzmann equation:

$$I/I_{max} = 1/\{1 + \exp[(V - V_h)/k]\},$$

where V is the conditioning potential, V_h is the voltage at which half the current is inactivated and k is the slope factor.

From four tested neurones, the application of 1 μM SKF38393 did not significantly modify the steady-state inactivation (Fig. 5*A* and *C*) since, before and after application of the D_1 agonist, values for V_h were $-43 \pm 2{\cdot}7$ and $-44{\cdot}5 \pm 3{\cdot}3$ mV (mean ± S.E.M.) and for k were $5{\cdot}8 \pm 0{\cdot}45$ and $4{\cdot}9 \pm 0{\cdot}1$, respectively (Fig. 5*A*).

Effects of the D_1 agonist on time-dependent inactivation parameters

The possibility of SKF38393 affecting the time-dependent inactivation of the sodium current was examined during sustained depolarizations at different potentials. Decay of the inward currents elicited from a holding potential of −80 mV to the test potential ranging from −20 to 10 mV,

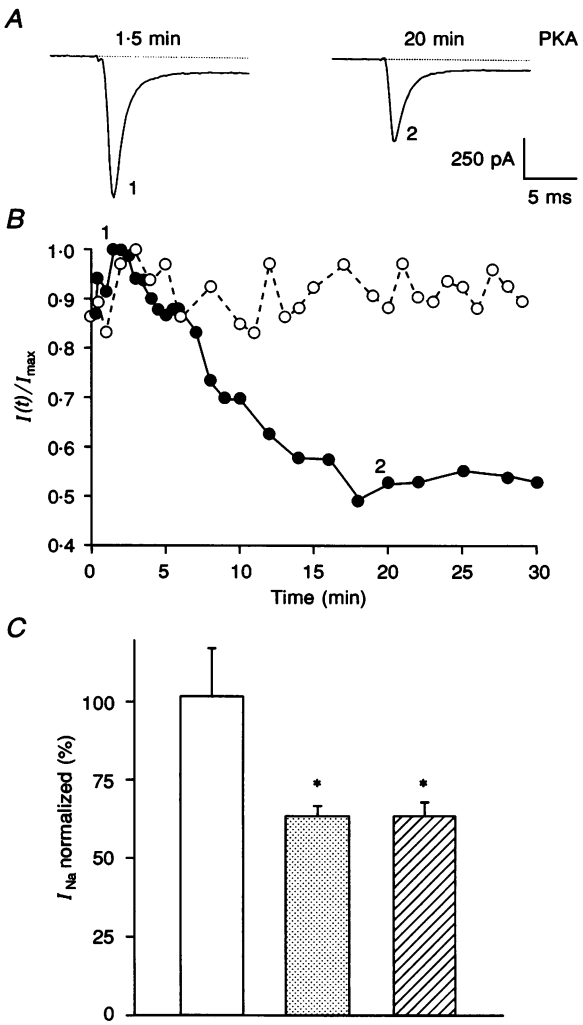

Figure 7. Intracellular dialysis of the catalytic subunit of PKA mimicked the effect of SKF38393

A and *B*, PKA time-dependently decreased the sodium current amplitude. The current traces 1–2 were recorded at the times indicated in part *B*, the whole-cell recording configuration was reached at time 0 (○, heat-inactivated PKA; ●, PKA). The heat-inactivated PKA had no significant effect. *C*, the normalized sodium current amplitude was plotted for neurones submitted to a SKF38393 application and for PKA-loaded neurones as compared to heat-inactivated PKA-loaded neurones; note that the reduction was of a similar extent (□, heat-inactivated PKA (3); ▩, PKA (13); ▨, SKF38393 (14)). Bars represent means ± S.E.M., with number of experiments in parentheses (*$P < 0{\cdot}005$ *versus* heat-inactivated PKA-loaded neurones was determined with a one-way analysis of variance, ANOVA, followed by a *post hoc* comparison using the Bonferroni test).

was fitted by a single exponential function. The inactivation time constants (τ_h) were determined by a least-squares curve fitting of the peak sodium current. This time constant was found to decrease with increasing depolarization (Fig. 6*B*). After the addition of SKF38393 (1 μM), τ_h was not significantly slowed as illustrated for a representative neurone during a voltage pulse to −10 mV (Fig. 6*A*). Before and after application of the D_1 agonist, τ_h values were 2·0 ± 0·4 and 2·0 ± 0·5 ms at −20 mV and 1·4 ± 0·2 and 1·5 ± 0·3 ms (means ± S.E.M.) at −10 mV, respectively ($n = 5$).

Another property of the sodium current investigated here, related to the inactivation characteristics described above, was the recovery from inactivation. Figure 6*C* shows an example of the sodium current elicited after varying intervals (Δt) between a prepulse and a test pulse of the same amplitude (from −80 to −20 mV). The rate of recovery from inactivation is represented in Fig. 6*D*. The peak amplitude of the sodium current evoked during the second pulse was normalized to that evoked during the first one and the resulting ratio was plotted as a function of time (Δt). As the duration of the recovery increased, the normalized current increased with a similar rate before and during application of SKF38393 (1 μM). The D_1 agonist did not significantly modify the rate for recovery from inactivation ($\Delta t_{½}$ was 3·0 ± 0·5 and 3·3 ± 0·5 ms before and after agonist application, respectively (means ± S.E.M., $n = 6$).

Does the D_1 agonist inhibit the sodium current by the cyclic AMP pathway?

Protein phosphorylation is a widespread mechanism for signal transduction and regulation in the nervous system (Walaas & Greengard, 1991). The rat brain α-subunit of the sodium channel has been reported to be phosphorylated either by the PKA or PKC, leading to a decrease in the sodium current amplitude (Numan, Catterall & Scheuer, 1991; Li, West, Lai, Scheuer & Catterall, 1992). Since the D_1 receptor is positively coupled to adenylate cyclase in striatal neurones (Stoof & Kebabian, 1981) and bath application of 8-bromo-cyclic AMP partially mimics the D_1 receptor-mediated effect on acutely dissociated striatal neurones (Surmeier *et al.* 1992), we studied the physiological effect of cyclic AMP-dependent phosphorylation on the amplitude of the sodium current. Thus to test for a role of PKA in sodium current inhibition produced by D_1 receptor activation, we employed either the catalytic subunit of PKA or a specific inhibitor.

Effects of the intracellular loading of the catalytic subunit of PKA

Cultured striatal neurones were recorded using internal solution containing the purified catalytic subunit of PKA (100 U ml^{-1}) and introduction into the cytoplasm was performed by simple diffusion from the pipette milieu to the cytosol. The results illustrated in Fig. 7 are from a

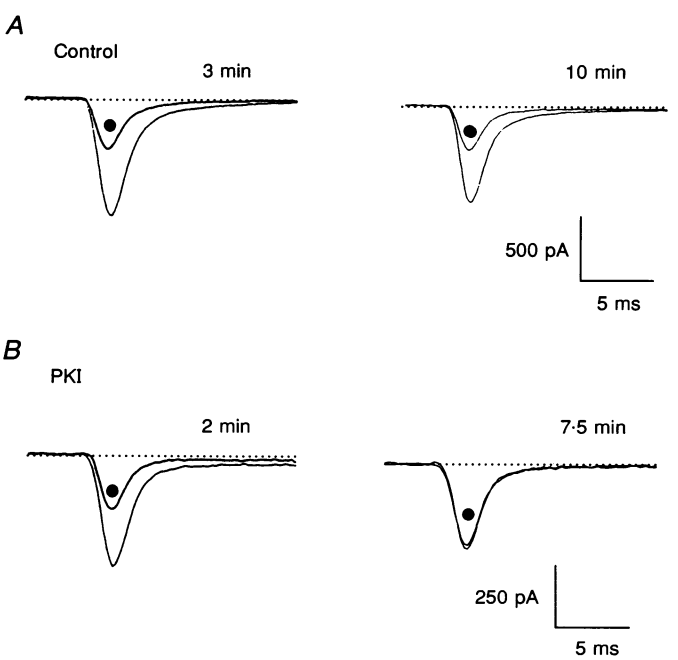

Figure 8. Intracellular dialysis of PKI blocked the effect of SKF38393

A, pairs of current traces evoked by 15 ms depolarizing pulses from −80 to −20 mV and recorded 2–3 min and 8–10 min after commencing whole-cell recordings, in the absence or presence (●) of 1 μM SKF38393. *B*, pairs of current traces obtained using the protocol described above with a patch pipette containing PKI (10 μM). Note that in contrast to the control conditions, SKF38393 was unable to decrease the amplitude of the sodium current during the second application. Current traces were recorded at the times indicated.

population study in which neurones were recorded using internal solution with PKA or with heat-inactivated PKA (30 min at 70 °C). The amplitude of the sodium current evoked by voltage pulses, from the holding potential of −80 to −20 mV, was followed during time after reaching the whole-cell recording configuration (Fig. 7*B*). In all tested PKA-loaded neurones, following approximately 4 min of recording to allow the protein to diffuse into the cytosol (see Fig. 7*B*), a time-dependent decrease of the sodium current amplitude was observed (Fig. 7*A* and *B*; $n = 13$). Interestingly, the reduction in current amplitude after complete loading (the mean maximal inhibition was $36{\cdot}7 \pm 3{\cdot}1\%$) was similar to the one obtained after application of SKF38393 ($37{\cdot}8 \pm 4{\cdot}9\%$, see above), demonstrating therefore that the intracellular dialysis of the catalytic subunit of the PKA was effective in reducing the sodium current to a similar extent to the D_1 agonist (Fig. 7*C*). Conversely, the sodium current amplitude was not significantly altered following loading of the neurones with a heat-inactivated PKA (Fig. 7*B* and *C*).

Effects of the intracellular dialysis of the pseudosubstrate inhibitor (PKI)

To test the involvement of the PKA in the D_1 receptor-mediated inhibition of sodium current more acutely and directly, a pseudosubstrate which irreversibly bound to the catalytic subunit of the PKA was used as a specific inhibitor of the kinase A (PKI 5–24). Using an intracellular solution containing 10 μM PKI 5–24, two different voltage-clamp protocols were performed. The first one consisted of two sequential applications of the D_1 agonist, once early after reaching the whole-cell configuration (2–3 min) and, after recovery, once again after reaching the whole-cell configuration (6–8 min) (Fig. 8). This allowed us to test the effect of SKF38393 before and after the complete loading of the neurone with the PKI. During the first application, SKF38393 depressed the amplitude of the sodium current in 57% of the tested neurones (4/7) (see Fig. 8*B*). This was similar to the results obtained in neurones dialysed with the standard intracellular solution (see above and Fig. 8*A*). Conversely, none of the four neurones which have responded to the D_1 agonist during the early application exhibited a reduction in the amplitude of the sodium current during the late application of this compound (Fig. 8*B*).

In a second protocol, the PKI-loaded neurones were subject to the application of SKF38393 only late (6–8 min) after attaining the whole-cell configuration. In these conditions, no neurone exhibited a response to the D_1 agonist ($n = 15$; data not shown) and this was clearly and significantly different from the 64% responding neurones under control conditions (see above). Therefore, these results demonstrated that the specific inhibition of PKA prevented the reduction in the sodium current amplitude mediated by the D_1 receptor.

DISCUSSION

Involvement of PKA in the D_1 receptor-induced inhibition of the voltage-gated sodium current

We reported that in cultured striatal neurones, the activation of the dopamine D_1 receptor by a specific agonist reduced the sodium current amplitude without alterations of either activation or inactivation properties. The modulation we described here appears therefore to be different from the one reported for the cardiac sodium channel which presents a high degree of voltage dependence (Schubert *et al.* 1989). This later modulation was indeed found to result partially from a membrane-delimited regulation involving activation of a β-adrenergic receptor coupled to the $G_{\alpha,s}$ protein. Such an effect can be extended to other voltage-gated ionic channels modulated by a membrane-delimited pathway (e.g. calcium or potassium channels) for which membrane-delimited modulation has been extensively demonstrated to be voltage dependent (Kasai, 1992).

Similarly, the involvement of arachidonic acid, which could be produced by striatal neurones in response to the activation of dopamine receptors (Piomelli, Pilon, Giros, Sokoloff, Martres & Schwartz, 1991), can be ruled out in our experiments since the depression in sodium current amplitude induced by arachidonic acid results from a shift in the steady-state inactivation to more hyperpolarized potentials (Fraser *et al.* 1993). This effect, which probably involves a putative fatty acid binding domain on sodium channels, remains to be elucidated.

The relatively slow kinetics of the action and reversal of D_1 agonist reported here seem, in fact, compatible with a phosphorylation–dephosphorylation mechanism. We have demonstrated precisely that the D_1 receptor-mediated effect on the striatal sodium channels involved the activation of PKA by mimicking its effect using intracellular dialysis of the catalytic subunit of PKA and by blocking using a pseudosubstrate of PKA (e.g. PKI). This suggested that, in striatal neurones, the sodium current flow through the channel phosphorylated by PKA is reduced in amplitude without alterations in activation and inactivation properties. Indeed, the rat brain sodium channel has been shown to be phosphorylated by PKA (Rossie & Catterall, 1987). Consistent with our observations, the PKA-mediated phosphorylation of the brain sodium channels, specifically the type IIA which are the predominant form expressed in the brain (Yarowski, Krueger, Olson & Clevinger, 1991), results in a reduction in the sodium current amplitude without any alterations of the activation or inactivation properties (Li *et al.* 1992). This decrease results from a reduced open probability of single channels during depolarization when they are phosphorylated by PKA (Li *et al.* 1992). We therefore

propose that, in striatal neurones, the PKA-mediated phosphorylation of the sodium channel, which brings negative charges to the internal side of the channel, reduces the open probability of the sodium channel without any changes in its voltage dependency.

Interestingly, the basal activity of PKA in neurones confers a basal level of phosphorylation to these sodium channels (Rossie & Catterall, 1987) leading to a reduction in their activity and therefore to a depression of the basal sodium current (Li *et al.* 1992). This suggests that this current could theoretically be either up- or down-regulated. This picture, however, is probably more complicated since it has been shown recently that this effect of PKA-mediated phosphorylation requires a concomitant phosphorylation of the channel Ser-1506 by PKC (Li, West, Numan, Murphy, Scheuer & Catterall, 1993). Therefore, although this remains to be elucidated, our results strongly suggest a basal phosphorylation of channel Ser-1506 by PKC in striatal neurones.

Physiological significance of the D_1 agonist-induced reduction of the sodium current in the striatum

In agreement with voltage-clamp experiments, our current-clamp study showed that the activation of the dopamine D_1 receptor decreased the excitability of striatal neurones by increasing the threshold for generation of action potentials. This confirms intracellular recordings of striatal neurones in brain slices, demonstrating that dopamine acting through the D_1 receptor inhibited the neuronal firing by increasing the threshold for generation of action potentials (Calabresi, Stanzione, Stefani, Mercuri & Bernardi, 1987). Using specific blockers of potassium, calcium and sodium channels, the involvement of a sodium conductance in the D_1 receptor-mediated effect has been suggested (Calabresi *et al.* 1987). Activation of the D_1 receptor also resulted in a decrease in excitatory synaptic potentials (Calabresi, Benedetti, Mercuri, Stanzione, Stefani & Bernardi, 1988). Finally, extracellular single unit recording demonstrated that iontophoretic application of the D_1 agonist mainly decreased the glutamate-evoked activation of the firing rate in striatal neurones (Hu & Wang, 1988). However, at low iontophoretic current, the same agonist was able to enhance this firing rate (Hu & Wang, 1988). This dual effect could be related to the differential action of a D_1 agonist on the excitatory amino acid-evoked neuronal excitation since SKF38393 potentiated the effect of the *N*-methyl-D-aspartate (NMDA) receptor activation and partly inhibited that of the non-NMDA receptors (Cepeda, Buchwald & Levine, 1993). Although this remains to be demonstrated, the involvement of modulations of potassium or calcium channels may account for this potentiation.

We did not observe consistent changes in the membrane potential during application of the D_1 agonist. This was also in agreement with the previous reports (Calabresi *et al.* 1987; Cepeda *et al.* 1993) in preparations lacking most of the synaptic afferents onto striatal neurones such as ours.

Although we did not characterize the recorded neurones on their immunohistochemical properties, the neurones exhibiting a response to the D_1 agonist probably belong mostly to the striato-nigral subpopulation of striatal neurones (Gerfen, 1992).

Altogether, our results and previous studies using different approaches (Calabresi *et al.* 1987; Calabresi *et al.* 1988; Hu & Wang, 1988; Surmeier *et al.* 1992) strongly suggest that the activation of the postsynaptic dopamine D_1 receptor depresses the excitability of striato-nigral neurones.

Albin, R. L., Young, A. B. & Penney, B. (1989). The functional anatomy of basal ganglia disorders. *Trends in Neurosciences* **12**, 366–375.

Brussaard, A. B., Lodder, J. C., Ter Maat, A., de Vlieger, T. A. & Kits, K. S. (1991). Inhibitory modulation by FMRFamide of the voltage-gated sodium current in identified neurones in *Lymnaea stagnalis*. *Journal of Physiology* **441**, 385–406.

Calabresi, P., Benedetti, M., Mercuri, N. B. & Bernardi, G. (1988). Endogenous dopamine and dopaminergic agonists modulate synaptic excitation in neostriatum: intracellular studies from naive and catecholamine-depleted rats. *Neuroscience* **27**, 145–157.

Calabresi, P., Mercuri, N. B., Stanzione, P., Stefani, A. & Bernardi, G. (1987). Intracellular studies on the dopamine-induced firing inhibition of neostriatal neurons *in vitro*: evidence for a D_1 receptor involvement. *Neuroscience* **20**, 757–771.

Catterall, W. A. (1992). Cellular and molecular biology of voltage-gated sodium channels. *Physiological Reviews* **72**, S15–48.

Cepeda, C., Buchwald, N. A. & Levine, M. S. (1993). Neuromodulatory actions of dopamine in the neostriatum are dependent upon the excitatory amino acid receptor subtypes activated. *Proceedings of the National Academy of Sciences of the USA* **90**, 9576–9580.

Eccles, J. C. (1964). *The Physiology of Synapses*. Springer, Berlin.

Fraser, D. D., Hoehn, K., Weiss, S. & MacVicar, B. A. (1993). Arachidonic acid inhibits sodium currents and synaptic transmission in cultured striatal neurons. *Neuron* **11**, 633–644.

Freedman, J. E. & Weight, F. F. (1988). Single potassium channels activated by D_2 dopamine receptors in acutely dissociated neurons from rat corpus striatum. *Proceedings of the National Academy of Sciences of the USA* **85**, 3618–3622.

Gerfen, C. R. (1992). The neostriatal mosaic: multiple levels of compartimental organization in the basal ganglia. *Annual Review of Neuroscience* **15**, 285–320.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hoehn, K., Watson, T. W. J. & MacVicar, B. A. (1993). Multiple calcium channels in acutely isolated rat neostriatal neurons. *Journal of Neuroscience* **13**, 1244–1257.

Hu, X.-T. & Wang, R. Y. (1988). Comparison of effects of D_1 and D_2 dopamine receptor agonists on neurons in the rat caudate putamen: an electrophysiological study. *Journal of Neuroscience* **8**, 4340–4348.

Kasai, H. (1992). Voltage- and time-dependent inhibition of neuronal calcium channels by a GTP-binding protein in a mammalian cell line. *Journal of Physiology* **448**, 189–209.

Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G. & Wittig, B. (1991). Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature* **353**, 43–48.

Li, M., West, J. W., Lai, Y., Scheuer, T. & Catterall, W. A. (1992). Functional modulation of brain sodium channels by cAMP-dependent phosphorylation. *Neuron* **8**, 1151–1159.

Li, M., West, J. W., Numan, R., Murphy, B. J., Scheuer, T. & Catterall, W. A. (1993). Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. *Science* **261**, 1439–1442.

Lledo, P.-M., Homburger, V., Bockaert, J. & Vincent, J.-D. (1992). Differential G protein-mediated coupling of D_2 dopamine receptors to K^+ and Ca^{2+} currents in rat anterior pituitary cells. *Neuron* **8**, 455–463.

Lopez-Barneo, J., Castellano, A. & Toledo-Aral, J. (1990). Thyrotropin-releasing-hormone (TRH) and its physiological metabolite TRH-OH inhibit Na^+ channel activity in mammalian septal neurons. *Proceedings of the National Academy of Sciences of the USA* **87**, 8150–8154.

Nicoll, R. A., Malenka, R. C. & Kauer, J. A. (1990). Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Pharmacological Reviews* **70**, 513–565.

Numan, R., Catterall, W. A. & Scheuer, T. (1991). Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science* **254**, 115–118.

O'Dowd, B. F. (1993). Structures of dopamine receptors. *Journal of Neurochemistry* **60**, 804–816.

Ogata, N. & Tatebayashi, H. (1990). Sodium current kinetics in freshly isolated neostriatal neurones of the adult guinea pig. *Pflügers Archiv* **416**, 594–603.

Piomelli, D., Pilon, C., Giros, B., Sokoloff, P., Martres, M.-P. & Schwartz, J.-C. (1991). Dopamine activation of the arachidonic acid cascade as a basis for D_1/D_2 receptor synergism. *Nature* **353**, 164–167.

Rossie, S. & Catterall, W. A. (1987). Cyclic AMP-dependent phosphorylation of voltage-sensitive sodium channels in primary cultures of rat brain neurons. *Journal of Biological Chemistry* **262**, 12735–12744.

Schiffmann, S. N., Lledo, P.-M. & Vincent, J.-D. (1994). Dopamine D_1 receptor modulation of voltage-gated sodium channels is mediated through activation of the cAMP-dependent protein kinase in rat striatal neurons. *Journal of Physiology* **475.P**, 149–150*P*.

Schubert, B., Vandongen, A. M. J., Kirsch, G. E. & Brown, A. M. (1989). β-Adrenergic inhibition of cardiac sodium channels by dual G protein pathway. *Science* **245**, 516–519.

Sibley, D. R. & Monsma, F. J. (1992). Molecular biology of dopamine receptors. *Trends in Pharmacological Sciences* **13**, 61–75.

Stack, J. & Surprenant, A. (1991). Dopamine actions on calcium currents, potassium currents and hormone release in rat melanotrophs. *Journal of Physiology* **439**, 37–58.

Stoof, J. C. & Kebabian, J. W. (1981). Opposing roles for D_1 and D_2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. *Nature* **294**, 366–368.

Strange, P. G. (1993). New insights into dopamine receptors in the central nervous system. *Neurochemistry International* **22**, 223–236.

Surmeier, D. J., Bargas, J. & Kitai, S. T. (1989). Two types of A-current differing in voltage-dependence are expressed by neostriatal neurons of the rat neostriatum. *Neuroscience Letters* **103**, 331–337.

Surmeier, D. J., Eberwine, J., Wilson, C. J., Cao, Y., Stefani, A. & Kitai, S. T. (1992). Dopamine receptor subtypes colocalize in rat striatonigral neurons. *Proceedings of the National Academy of Sciences of the USA* **89**, 10178–10182.

Surmeier, D. J., Kita, H. & Kitai, S. T. (1988). The expression of γ-aminobutyric acid and Leu-enkephalin immunoreactivity in primary monolayer cultures of rat striatum. *Developmental Brain Research* **42**, 265–282.

Surmeier, D. J., Stefani, A., Foehring, R. C. & Kitai, S. T. (1991). Developmental regulation of a slowly-inactivating potassium conductance in rat neostriatal neurons. *Neuroscience Letters* **122**, 41–46.

Walaas, S. I. & Greengard, P. (1991). Protein phosphorylation and neuronal function. *Pharmacological Reviews* **43**, 299–349.

Woodward, R. M., Panicker, M. M. & Miledi, R. (1992). Actions of dopamine and dopaminergic drugs on cloned serotonin receptors expressed in *Xenopus* oocytes. *Proceedings of the National Academy of Sciences of the USA* **89**, 4708–4712.

Yarowsky, P. J., Krueger, B. K., Olson, E. & Clevinger, E. C. (1991). Brain and heart sodium channel subtype mRNA expression in rat cerebral cortex. *Proceedings of the National Academy of Sciences of the USA* **88**, 9453–9457.

Acknowledgements

We thank Stéphane Pere for fine technical assistance and Clyve D'Santos who carefully scrutinized the English. S.N.S. was supported by a fellowship from the CNRS, France and is a Research Associate of the FNRS, Belgium. This work was supported by grants from CNRS, DRET (Direction de la Recherche et Technique), FMRE (Belgium) and PAI (Pôle D'Attraction Interuniversitaire) (Belgium).

Received 14 March 1994; accepted 13 July 1994.