Phosphorylation- and voltage-dependent inhibition of neuronal calcium currents by activation of human D_{2(short)} dopamine receptors

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1 Activation of human D_{2(s)} dopamine receptors with quinpirole (10 nM) inhibits ω -conotoxin GVIA-sensitive, high-threshold calcium currents when expressed in differentiated NG108-15 cells (55% inhibition at +10 mV). This inhibition was made irreversible following intracellular dialysis with the non-hydrolysable guanosine triphosphate analogue GTP- γ -S (100 μ M), and was prevented by pretreatment with pertussis toxin (1 μ g ml⁻¹ for 24 h).

2 Stimulation of protein kinase C with the diacylglycerol analogue, 1-oleoyl-2-acetyl-sn-glycerol (100 μ M), also attenuated the inhibition of the sustained calcium current but did not affect the receptor-mediated decrease in rate of current activation. Similarly, okadaic acid (100 nM), a protein phosphatase 1/2A inhibitor, selectively occluded the inhibition of the sustained current.

3 The depression of calcium currents by quinpirole (10 nM) was enhanced following intracellular dialysis with 100 μ M cyclic adenosine monophosphate (cyclic AMP, 72.8 $\pm$ 9.8% depression), but was not mimicked by the membrane permeant cyclic GMP analogue, Sp-8-bromoguanosine-3',5':cyclic monophosphorothioate (100 μ M).

4 Inhibition of calcium currents was only partly attenuated by 100 ms depolarizing prepulses to +100 mV immediately preceding the test pulse. However, following occlusion of the sustained depression with okadaic acid (100 nM) the residual kinetic slowing was reversed in a voltage-dependent manner ($P < 0.05$).

5 Thus pertussis toxin-sensitive G-proteins liberated upon activation of human D_{2(short)} dopamine receptors inhibited high-threshold calcium currents in two distinct ways. The decrease in rate of calcium current activation involved a voltage-dependent pathway, whereas the sustained inhibition of calcium current involved, in part, the voltage-resistant phosphorylation by cyclic AMP-dependent protein kinases and subsequent dephosphorylation by protein phosphatases 1/2A.

Keywords: Calcium channels; D₂ dopamine receptor; G-proteins; neuronal calcium currents; cyclic nucleotides; protein kinases

Introduction

Dopamine receptors regulate the function of calcium currents in several cell types. One of the best characterized are rat pituitary melanotrophs in which low-threshold, and dihydropyridine sensitive high-threshold calcium currents are inhibited by activation of D₂ dopamine receptors (Lledo *et al.*, 1990; Keja *et al.*, 1992; Nussinovitch & Kleinhaus, 1992; Seabrook *et al.*, 1994a). Since the discovery that the D₂-like dopamine receptor family is composed of multiple structural subtypes, including several isoforms of the D₂, D₃, and D₄ subtypes, much interest has been generated concerning possible differences in the functional properties of these subtypes both within brain tissue and at the cellular level (reviewed in Grandy & Civelli, 1992; Schwartz *et al.*, 1992). However there are few, if any, ligands available that can discriminate between the short and long isoforms of the D₂ receptor subtype (Leysen *et al.*, 1993; however see also Castro & Strange, 1993), and this has made a detailed examination of their functional properties difficult. The separate transfection of receptor cDNA's into heterologous cell lines permits studies that are independent of other receptor isoforms, and furthermore allows the characterization of the functional properties of human receptors. Consequently we have transfected the short isoform of the human D₂ dopamine receptor into neuroblastoma x glioma NG108-15 cells (Seabrook *et al.*, 1994b). The present study was carried out to determine the mechanism(s) by which

D_{2(short)} receptors regulate the function of neuronal calcium channels that are not present in pituitary melanotrophs.

In mammalian brain, the release of dopamine is co-operatively dependent upon calcium influx into nerve terminals (Nachshen & Sanchez-Armass, 1987), and this occurs mainly through ω -conotoxin GVIA-sensitive calcium channels (Herdon & Nahorski, 1989). The behavioural effects of stimulation of D₂ receptors are in many cases accentuated by the co-application of D₁ receptor agonists (reviewed in Clark & White, 1987; LaHoste & Marshall, 1993). This synergy is paradoxical considering that D₁ and D₂ receptors have stimulatory and inhibitory effects respectively on adenylate cyclase activity (Kebabian & Calne, 1979). However, Piomelli and colleagues (1991) have recently shown that arachidonic acid release following D₂ receptor activation is also potentiated by D₁ receptor stimulation via an increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) production. Whether the inhibition of neuronal calcium currents by D₂ receptor activation is similarly modulated by cyclic AMP remains to be determined.

When differentiated into a neuronal phenotype, neuroblastoma x glioma NG108-15 cells express at least three types of calcium current. These can be distinguished by their voltage-dependence of activation into a low-threshold T-type, and at least two high-threshold subtypes (Fishman & Spector, 1981; Hering *et al.*, 1985; Docherty, 1988). The latter include dihydropyridine resistant N-type currents that are blocked by ω -conotoxin GVIA (Caulfield *et al.*, 1992; Kasai & Neher, 1992; Seabrook *et al.*, 1994b). These high-threshold currents are depressed by activation of various endogenous G-protein

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linked receptors such as opiate, muscarinic, and somatostatin receptors (Tsunoo *et al.*, 1986; McFadzean & Docherty, 1989; Caulfield *et al.*, 1992). This neurotransmitter regulation of calcium channels typically involves pertussis toxin sensitive G-proteins such as G_{α_o} subunits (reviewed in Scott *et al.*, 1991; Hille 1992; McCleskey, 1994), but also in some cases their $\beta\gamma$ counterparts (Kleuss *et al.*, 1991). Furthermore the second messenger systems involved may act via membrane delimited and/or by cytosolic pathways through protein phosphorylation (Hille, 1992). Consistent with this the pore forming α_1 subunit of calcium channels can be phosphorylated by several protein kinases, including protein kinase A and C, the actions of which can be terminated by dephosphorylation with protein phosphatases 1 or 2 (Hell *et al.*, 1994; Zhao *et al.*, 1994). To investigate the role of G-proteins and protein phosphorylation in the function of human $D_{2(\text{short})}$ dopamine receptors, this study examined the involvement of cyclic nucleotides as well as protein kinases in receptor mediated inhibition of N-type calcium currents in transfected neuroblastoma cells.

Methods

Calcium currents were recorded from transfected NG108-15 cells by methods described previously (Seabrook *et al.*, 1994b).

Cells were grown in tissue culture in a medium that contained Dulbecco's Modified Eagles Medium (GIBCO), 10% foetal calf serum, 2 mM L-glutamine (Geneticin), and were plated on poly-L-lysine coated coverslips. Cells were differentiated for 3 to 5 days using 10 μM prostaglandin E_1 and 50 μM isobutylmethylxanthine (Docherty, 1988). Whole cell patch clamp recordings were made with borosilicate glass microelectrodes (Clark Electromedical Instruments) which were filled with a solution that contained (in mM): CsCl 110, tetraethylammonium.Cl 25, EGTA 10, HEPES 40 and MgCl_2 3, adjusted to pH 7.3 with CsOH. Cells were perfused at a rate of 1 to 2 ml min^{-1} with an extracellular solution that contained (in mM): NaCl 135, glucose 10, HEPES 10, KCl 5, CaCl_2 or BaCl_2 5 and tetrodotoxin 0.5 μM , pH 7.4, at 22°C. Drugs were applied via the superfusion system. Nystatin perforated patch clamp recordings were made using a pipette solution that contained (in mM): $\text{CsH}_2\text{O}_3\text{SCs}$ 100, CsCl 25, MgCl_2 3, HEPES 40, adjusted to pH 7.3 with CsOH. Cells were voltage-clamped and calcium currents were elicited by 100 ms depolarizing pulses from -60 to $+10$ mV at 0.033 Hz. Currents were amplified with an Axopatch 200 patch clamp amplifier (Axon Instruments), and captured on line via a CED1401 interface (Cambridge Electronic Design) which was connected to a Compaq 486 microcomputer. Linear leak currents were subtracted with the amplifier, and by the CED voltage-clamp

Figure 1 Persistence of D_2 receptor inhibition of calcium currents following inhibition of L-type currents with nisoldipine, chelation of $[\text{Ca}^{2+}]_i$ with EGTA, and following intracellular dialysis with the non-hydrolysable GTP analogue, GTP- γ -S. (a) Superimposed calcium currents in control conditions and following equilibration in nisoldipine (10 μM) and quinpirole (10 nM). Lower trace represents the change in membrane holding potential during a 100 ms pulse. Calibration bar = 500 pA. (b) Histogram depicting the maximal inhibition of the high-threshold calcium current obtained at $+10$ mV using nystatin-perforated patch clamp recording ($n=21$), whole cell recording with electrodes containing 10 mM EGTA ($n=5$), and with 100 μM GTP- γ -S ($n=4$). Following pretreatment with pertussis toxin (PTX, 1 $\mu\text{g ml}^{-1}$ 24 h) the responses to D_2 receptor activation were abolished ($n=4$). (c) Superimposed currents following internal dialysis with GTP- γ -S (100 μM , control) demonstrating the slowing in rate of activation of current with quinpirole (10 nM), and its lack of reversal with [GTP- γ -S] following wash off. Calibration bar = 400 pA. (d) Time course of irreversible inhibition with quinpirole (10 nM applied for duration of horizontal line) following treatment with GTP- γ -S (100 μM), data are pooled from 4 cells (cf. Figure 3).

analysis software using a P:N leak subtraction protocol (5:1). The series resistance in nystatin perforated patch clamp recordings (typically 10–20 MΩ cf. whole cell 5–10 MΩ) would introduce a clamp error of up to ± 5 mV assuming a maximum inward current of 500 pA, but series resistance compensation was not used in the present study to minimize noise. Currents were filtered at either 2 or 5 kHz high frequency cut off. Data are expressed as the mean $\pm$ s.e.mean, and unless stated percentages are the calcium current inhibition obtained with quinpirole (10 nM) normalized to the control current amplitude. Statistical analysis was carried out by analysis of variance in Microsoft Excel (version 5.0).

Drugs were obtained from the following companies: ethylene glycol bis(β -aminoethyl ether)-*N,N,N,N*-tetraacetic acid (EGTA), adenosine 3':5'-cyclic monophosphate (cyclic AMP), guanosine 5'-O-[3-thiotriphosphate] (GTP- γ -S), ω -conotoxin GVIA, and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) were purchased from Sigma. Quinpirole was purchased from Research Biochemical Inc. The Sp (Sp-8-Br-cGMPs) and Rp isomers (Rp-8-Br-cGMPs) of 8-bromoguanosine-3',5':cyclic monophosphorothioate sodium salt were purchased from Biolog Life Sciences Institute. Okadaic acid and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Calbiochem. Nisoldipine was obtained from Miles Pharmaceuticals. Drugs were dissolved either in distilled water, or as in the case of nisoldipine in dimethylsulphoxide (DMSO) with a final bath concentration of $<0.1\%$ DMSO which alone had no effect upon calcium current amplitudes.

Results

Calcium currents were recorded from 157 cells to which drugs were applied, by either nystatin-perforated or conventional whole cell techniques. Depolarizations from a holding potential of -100 mV to greater than -50 mV elicited a biphasic inward current. This consisted of a low-threshold (at -30 mV) transient current that was completely inactivated within a 200 ms pulse, and a non-inactivating high-threshold current. Cells had a mean capacitance of 78.6 ± 4.3 pF, and a high-threshold current density of 12.2 ± 3.4 pA pF $^{-1}$ at $+10$ mV ($n=12$). In nystatin perforated patch recordings this high-threshold current (-60 to $+10$ mV) was maximally depressed by $55.3 \pm 4.0\%$ ($n=21$) following perfusion with the D₂-like dopamine receptor agonist, quinpirole (10 nM). This effect was restricted to the high-threshold current in that no depression of low-threshold currents was observed (Seabrook *et al.*, 1994b). Furthermore the depression was still observed after inhibition of L-type calcium currents with the dihydropyridine, nisoldipine (10 μ M). In the presence of nisoldipine, quinpirole (10 nM) caused a $36.6 \pm 7.0\%$ ($n=5$) depression of the peak calcium current (Figure 1a). Co-application of nisoldipine and ω -conotoxin GVIA (100 nM) inhibited $>95\%$ of the inward current under these recording conditions ($n=4$).

Involvement of pertussis toxin-sensitive G-proteins but not $[Ca^{2+}]_i$

In conventional whole cell recordings, in which EGTA (10 mM) was included in the patch pipette to chelate intracellular calcium, no change in the maximal inhibition obtained following D₂ receptor activation was observed compared to experiments using nystatin-perforated patch recording (Figure 1). However, when extracellular Ba $^{2+}$ ions (5 mM) were substituted as the charge carrier in place of $[Ca^{2+}]_o$, the percentage inhibition of the peak current following D₂ receptor activation was accentuated from 55% to $79.9 \pm 7.6\%$ ($P < 0.001$; $n=9$). Consistent with the involvement of G-proteins, the effects of quinpirole were rendered irreversible when the non-hydrolysable GTP analogue GTP- γ -S (100 μ M, $n=4$) was included in the patch pipette (Figure 1). Furthermore responses were blocked by pretreatment of cells with pertussis toxin (1 μ g ml $^{-1}$ for 24 h; Figure 1) which se-

lectively ADP ribosylates G-proteins of the G_i and G_o subtype.

Role of protein phosphorylation

When cells were dialysed, via the patch pipette, with the diacylglycerol analogue, OAG (100 μ M) to activate protein kinase C, the depression of the high-threshold calcium current following the subsequent activation of D₂ receptors was attenuated (Figure 2). This effect was most evident on the sustained current (90 ms into the voltage step) where a $18.0 \pm 9.8\%$ depression was observed, compared to control values of $39.3 \pm 7.5\%$ ($P < 0.05$, $n=5$). In contrast, no significant attenuation of the depression of the peak current (10 ms into the voltage step) was observed ($27.4 \pm 6.3\%$, $n=5$).

Inclusion of the cyclic nucleotide, cyclic AMP (100 μ M) in the recording electrode potentiated the inhibition observed following D₂ receptor activation (Figure 3). This effect was fully reversible, and was evident on both the peak ($72.8 \pm 9.8\%$, $P < 0.01$, $n=5$) and sustained calcium current ($70.4 \pm 14.8\%$, $P < 0.01$, $n=5$). This increase in depression was not associated with the potentiation of a 'D₂ susceptible current' because the current density in 100 μ M cyclic AMP was unaffected (7.06 ± 1.57 pA pF $^{-1}$, $n=5$) compared to the control in the same batch of cells (7.20 ± 2.90 pA pF $^{-1}$, $n=5$). Furthermore forskolin (10 μ M), an irreversible activator of adenylate cyclase, did not alter the amplitude of whole cell

Figure 2 Modulation of D₂ receptor inhibition of calcium currents by diacylglycerol. (a) Superimposed calcium currents measured after intracellular dialysis with 100 μ M OAG (control) and following application of quinpirole (10 nM). Calibration bar = 1 nA. (b) Histogram comparing the inhibition of the peak and sustained components of the calcium current (see methods) with quinpirole under control conditions (open columns, $n=6$), and with 100 μ M [OAG]_i (shaded columns, $n=5$). The depression of the peak current was unaffected, whereas the inhibition of the sustained current was significantly reduced ($P < 0.05$).

calcium currents in these cells (see also Kasai, 1991). In contrast to the effects of cyclic AMP, extracellular application of the membrane permeant cyclic GMP analogue, Sp-8-bromoguanosine-3',5':cyclic monophosphorothioate ($100\text{ }\mu\text{M}$ for 2 min, $n=4$) did not mimic the effect of D_2 receptor activation. Consistent with the lack of involvement of cyclic GMP-dependent protein kinases, the inhibition caused by quinpirole was not blocked by the cyclic GMP-dependent protein kinase inhibitor Rp-8-Br-cGMP ($100\text{ }\mu\text{M}$).

To test further for the involvement of channel phosphorylation the effect of okadaic acid, a protein phosphatase inhibitor, was examined. Prevention of channel dephosphorylation by including okadaic acid (100 nM) in the patch pipette significantly ($P<0.01$) occluded the depression of the sustained current but not that of the peak current (Figure 3). Furthermore this treatment selectively unmasked the decrease in rate of calcium current activation that is superimposed on the sustained calcium current inhibition in control conditions.

The voltage-dependence of calcium current regulation by D_2 receptor activation was also examined by voltage-clamping cells at -60 mV , and immediately preceding the test pulse a depolarization to $+100\text{ mV}$ was applied (Figure 4). Alone this prepulse had no effect on the amplitude of calcium currents elicited by subsequent potential steps to $+10\text{ mV}$. Although the sustained inhibition following D_2 receptor activation was

partly relieved with these depolarizing prepulses ($P<0.05$, $n=7$), the decrease in rate of current activation was almost entirely voltage-dependent in that the transient inhibition observed when cells had been dialysed with okadaic acid was reversed by these prepulses (Figure 4).

Discussion

In neurones several signal transduction pathways are regulated by the activity of D_2 dopamine receptors. These include the inhibition of adenylate cyclase activity, potentiation of arachidonic acid release, activation of potassium currents, and decrease in calcium channel activity (reviewed in Grandy & Civelli, 1992; Schwartz *et al.*, 1992). However, the relative importance of each pathway in modulating cellular excitability and consequently the function of discrete brain regions, depends not only upon the tissue studied but also the neurotransmitter system that is modified. In this study we investigated the influence of D_2 dopamine receptors on neuronal calcium currents because it is this channel subtype that regulates dopamine release within the mammalian striatum (Herdon & Nahorski, 1989).

Activation of human $\text{D}_{2(\text{short})}$ dopamine receptors expressed in NG108-15 cells selectively inhibits the ω -conotoxin GVIA-sensitive high-threshold calcium current, a component of

Figure 3 Potentiation of D_2 receptor depression of calcium currents by elevation of intracellular cyclic AMP, and the selective occlusion of the sustained depression by inhibition of protein phosphatase activity with okadaic acid. (a) Superimposed calcium currents recorded after dialysis with $100\text{ }\mu\text{M}$ cyclic AMP (control), following equilibration in quinpirole (10 nM), and after washing for 2 min. Calibration bar = 1 nA . (b) Time course of depression of calcium currents with quinpirole demonstrating that the potentiation of the responses to quinpirole with [cyclic AMP] $_i$ was readily reversible. Data are pooled from 5 cells. (c) Superimposed whole cell calcium currents elicited after equilibration in 100 nM okadaic acid (control) and following perfusion with quinpirole (10 nM). Calibration bar = 1 nA . (d) Histogram depicting increase in both the peak and sustained inhibition of calcium currents by $100\text{ }\mu\text{M}$ [cyclic AMP] $_i$ ($n=5$), and the selective attenuation of the depression of the sustained calcium current with 100 nM okadaic acid ($n=4$). Asterisks denote significant difference at $P<0.01$ from control.

which is also attenuated by dihydropyridines (Seabrook *et al.*, 1994b). The proportion of the high-threshold current inhibited by D₂ receptors in differentiated NG108-15 cells was much greater than that observed in D₂ transfected GH₄C₁ pituitary cells (Seabrook *et al.*, 1994a,b). This is most likely due to the low density, if any, of dihydropyridine resistant N-type calcium channels in GH₄C₁ cells. In the present study two components to the inhibition of ω -conotoxin GVIA-sensitive high-threshold calcium current were observed. These involved a decrease in rate of current activation (type 1) and the steady state depression of the sustained current at the end of a 100 ms voltage step (type 2). Both type 1 and type 2 depression were evident on N-type calcium currents that were recorded in the presence of nisoldipine, a dihydropyridine calcium channel antagonist. A decrease in rate of calcium current activation has also been described following activation of other receptor subtypes including GABA_B receptors (Scott *et al.*, 1991) which are known to couple by signal transduction mechanisms similar to D₂-like dopamine receptors on midbrain dopamine neurones (Lacey *et al.*, 1987). In rat dorsal root ganglia neurones, GABA_B receptors inhibit calcium channels via α_o G-protein subunits (Campbell *et al.*, 1993). Similarly, inhibition of L-type calcium channels in pituitary cells by D₂ receptors

can be blocked by antibodies and antisense oligonucleotides designed against α_o G-protein subunits (Lledo *et al.*, 1992). Consistent with the direct involvement of G-proteins in D₂ receptor inhibition of N-type calcium channels in NG108-15 cells, responses to quinpirole were rendered irreversible by the non-hydrolysable GTP analogue GTP- γ -S and were inhibited by pertussis toxin.

D₂ dopamine receptors can also activate α_i G-protein subunits which in turn inhibit adenylate cyclase activity (e.g. Kebabian & Calne, 1979). This enzyme catalyses the production of cyclic AMP from ATP, and thus activation of D₂ receptors can decrease intracellular cyclic AMP levels. To exclude the possibility that inhibition of calcium currents was due to a reduction in [cyclic AMP]_i, the effect of raising [cyclic AMP]_i by including it in the patch pipette was examined. However, instead of masking the effects of D₂ receptor activation, raised [cyclic AMP]_i increased the proportion of the calcium current that was depressed. Consequently inhibition of adenylate cyclase activity was not the mechanism by which D₂ receptors depress calcium currents in these cells. Stimulation of cyclic AMP-dependent protein kinases has been shown to increase L-type calcium currents (reviewed in Hescheler *et al.*, 1990), suggesting that cyclic AMP may be increasing the proportion

Figure 4 Voltage-dependence of calcium current inhibition under control conditions, and after the selective depression of the sustained inhibition with intracellular perfusion with okadaic acid. (a) Superimposed calcium currents recorded under control conditions (1) and following a 100 ms prepulse to +100 mV, and in the presence of 10 nM quinpirole (3) and following the +100 mV prepulse (4). Note that both the outward current elicited at +100 mV, which is probably due to the outward flux of Cs⁺ ions through calcium channels, and the inward calcium currents are completely inhibited by bath application of 100 μ M CdCl₂. Lower trace represents superimposed holding potentials used to elicit the currents described above, calibration bar = 2 nA. (b) Histogram summarising the voltage-dependence of D₂-receptor mediated inhibition of calcium currents under control conditions. The prepulse alone did not affect the amplitude of the calcium current, but significantly reduced the depression, albeit incompletely, caused by 10 nM quinpirole ($n=7$, $P<0.01$). (c) Reversal of the decrease in rate of calcium current activation, that was unmasked following treatment with 100 nM okadaic acid, with prepulses to +100 mV. Annotations are the same as in (a). Capacitance transients are blanked for clarity. (d) Histogram depicting the mean depression with quinpirole (10 nM) under control conditions and with a prepulse to +100 mV. Note that the rundown of the calcium current was more accentuated after treatment with okadaic acid, and that the slowing in rate of current activation was fully reversed with the depolarizing prepulse compared to the recovery. * $P<0.05$; NS = non-significant.

of the D_2 -sensitive current. However, this cannot explain our data because the principal effect of D_2 receptor activation was on the dihydropyridine-insensitive high-threshold current, and the calcium current density in cyclic AMP dialysed cells was not significantly affected. Furthermore, in whole cell patch clamp studies where adenylate cyclase was directly activated with forskolin the amplitude of N-type calcium current was unaffected (Kasai 1991; this study). This insensitivity to forskolin contrasts with the effect of directly increasing [cyclic AMP]_i levels which potentiated the calcium current inhibition by D_2 receptors. This may be a consequence of either forskolin generating less [cyclic AMP]_i during relatively short incubation periods (< 10 min), the effects of channel phosphorylation being evident only on channels that have been modified by G-protein subunits following D_2 receptor stimulation, or alternatively to a direct action of cyclic AMP on D_2 receptors or its associated signal transduction pathway(s). N-type calcium channel $\alpha 1$ subunits are indeed substrates for phosphorylation by cyclic AMP-dependent protein kinase A (Hell *et al.*, 1994). Thus potentiation of responses to D_2 receptor activation with elevated [cyclic AMP]_i may involve direct phosphorylation of channels by PKA. Irrespective of the precise mechanisms involved, potentiation of D_2 receptor-mediated inhibition of calcium channel function with [cyclic AMP]_i may help explain the paradoxical functional synergism between these receptors and the D_1 dopamine subtype (Clark & White, 1987; Momiyama *et al.*, 1993), which are known to have opposing effects on adenylate cyclase activity (Kebabian & Calne, 1979). To test further the hypothesis that phosphorylation was necessary for D_2 receptor-mediated inhibition of N-type calcium currents, the effects of stimulating protein kinase C and inhibition of protein phosphatase activity were examined.

It was unlikely that $[Ca^{2+}]_i$ mobilisation was a critical determinant of calcium current regulation by D_2 receptor activation in NG108-15 cells because chelation of $[Ca^{2+}]_i$ with EGTA did not block this effect. Likewise, substitution of Ca^{2+} ions with Ba^{2+} as the charge carrier did not prevent the inhibition of calcium currents but actually enhanced it, a phenomenon that was probably due to the removal of calcium-dependent inactivation (Kalman *et al.*, 1991). Further evidence that D_2 receptor inhibition of calcium currents did not involve $[Ca^{2+}]_i$ mobilisation was that quinpirole (10 nM to 10 μ M) had no effects upon $[Ca^{2+}]_i$ levels, which was studied using FURA-2 microspectrofluorimetry in the same clonal cell line (unpublished observations; however cf. rat D_2 receptors in Castellano *et al.*, 1993). Calcium-dependent enzymes, such as protein kinase C, have also been implicated in the regulation of calcium channel activity (Rane & Dunlap, 1986; 1990). Thus to test for its involvement the action of the diacylglycerol analogue OAG was examined (Gilmore & Martin, 1983; Nishizuka, 1984). When calcium was used as the charge carrier the sustained depression of calcium currents (type 2 inhibition) was selectively attenuated by pretreatment with OAG, without any effect on the D_2 receptor mediated decrease in rate of calcium current activation (type 1). Thus under certain circumstances calcium influx through channels in NG108-15 cells may contribute to N-type calcium current inhibition via PKC activation.

D_2 dopamine receptors can also potentiate phospholipase A_2 (PLA₂) activity in a cyclic AMP-dependent manner (Kanterman *et al.*, 1991). PLA₂ catalyses the hydrolysis of phospholipids to produce arachidonic acid which can directly modulate ion channel activity. However, PLA₂, like PKC, is a Ca^{2+} -dependent enzyme and alone arachidonic acid does not modulate N-type calcium current activity in these cells (Kasai, 1991). Other kinases including cyclic GMP-dependent protein kinases have also been implicated in neurotransmitter receptor modulation of calcium channel function (Meriney *et al.*, 1994). No evidence for this pathway in regulating N-type calcium currents in NG108-15 cells was found because these currents were unaffected by either the membrane permeant cyclic GMP analogue Sp-8-Br-cGMP, or by the cyclic GMP antagonist Rp-8-Br-cGMP.

Thus D_2 receptors inhibit calcium currents in NG108-15 cells by mechanism(s) that do not involve inhibition of adenylate cyclase activity, calcium mobilisation, or potentiation of arachidonic acid release, but that under certain circumstances is dependent upon channel phosphorylation by PKC, and cyclic AMP-dependent protein kinases. The involvement of channel phosphorylation was confirmed by the ability of okadaic acid to occlude the inhibition of the sustained calcium current following receptor activation. Okadaic acid selectively inhibits protein phosphatases 1/2A and thus can prevent protein dephosphorylation. Under circumstances where there are background levels of phosphorylation, inhibition of phosphatase activity would increase the number of channels that were phosphorylated and hence inactivated. Therefore control calcium currents in okadaic acid would already be partly depressed. Consistent with this no sustained inhibition of the high-threshold calcium current was observed with quinpirole after treatment with intracellular okadaic acid. Furthermore, okadaic acid selectively isolated the kinetic slowing that was associated with receptor activation. Thus type 1 and type 2 inhibition of N-type calcium currents were mediated by two distinct processes. It was possible to distinguish further between these components by their voltage-dependence.

Neurotransmitter receptor modulation of N- and L-type calcium currents in neurones of sympathetic ganglia involves both cytosolic and membrane delimited pathways (Beech *et al.*, 1992; Hille, 1992). These pathways are distinguished into components that are voltage-dependent and voltage-resistant in that depolarizing prepulses applied immediately preceding the test pulse can relieve the inhibition. In differentiated NG108-15 cells the decrease in rate of activation of N-type calcium currents caused by activation of endogenous opiate receptors can also be reversed in a voltage-dependent manner (Kasai, 1992). Likewise in the present study the kinetic slowing of calcium currents caused by D_2 receptors, which was unmasked following pretreatment with okadaic acid, could be reversed by depolarizing prepulses to +100 mV. In contrast, the sustained inhibition of calcium currents was only partly reduced by these prepulses and thus was voltage-resistant. The term voltage-resistant is used here intentionally because it is known that disruption of the direct interaction of G-proteins with calcium channels by positive membrane potentials undergoes a time-dependent relaxation when the membrane is repolarized to negative values. However, unlike noradrenoceptor inhibition of calcium currents in sympathetic neurones (Beech *et al.*, 1992), both the sustained depression and kinetic slowing following D_2 receptor activation in NG108-15 cells involved pertussis toxin-sensitive G-proteins.

The importance of protein phosphorylation for the inhibition of ω -conotoxin GVIA-sensitive calcium currents by D_2 receptors in NG108-15 cells contrasts with the effects of phosphorylation on dihydropyridine-sensitive calcium channels. Phosphorylation of these calcium channel α subunits, for example by cyclic AMP-dependent PKA, increases single channel activity and can thus potentiate the amplitude of macroscopic L-type calcium currents (reviewed in Dolphin, 1995). In sympathetic neurones, PKC activation also precludes neurotransmitter-mediated inhibition of N-type calcium currents (Rane & Dunlap, 1986; 1990). These latter findings have been considered controversial (reviewed by Scott *et al.*, 1991; Dolphin, 1995). But combined with results from the present study this suggests that the importance of phosphorylation, whether inhibitory by increasing the susceptibility of calcium currents to inhibition by G-proteins, or excitatory, depends upon the calcium channel subtype that is affected.

In conclusion, this study demonstrates that the short isoform of the human D_2 dopamine receptor can selectively inhibit N-type calcium currents in differentiated NG108-15 cells. Modulation of these calcium channel currents was mediated via pertussis toxin-sensitive G-proteins and involved two distinct pathways. The decrease in rate of calcium current activation (type 1) involved a voltage-dependent pathway, whereas the sustained inhibition of the calcium current (type 2) in-

volved, in part, the voltage-resistant phosphorylation by cyclic AMP-dependent protein kinases and dephosphorylation by protein phosphatases 1/2A. The accentuation of D₂ receptor-mediated inhibition of neuronal calcium currents by cyclic AMP may help explain how D₁ receptor activation can potentiate the effects of D₂ receptor agonists in brain.

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