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Fluspirilene block of N-type calcium current in NGF-differentiated PC12 cells

[1,*]C.J. Grantham, M.J. Main & M.B. Cannell

Department of Pharmacology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE and *Lilly Research Centre Ltd., Erl Wood Manor, Windlesham, Surrey GU20 6PH

1 High voltage-activated calcium currents were recorded in nerve growth factor (NGF)-differentiated PC12 cells with the whole-cell patch clamp technique. After exposure to NGF for 3–10 days the PC12 cells developed neurone-like processes and calcium currents which were pharmacologically separable into L- and N-types (defined by sensitivity to nifedipine and ω-conotoxin GVIA respectively).

2 After blocking the L-type calcium channels with nifedipine (10 μM), ω-conotoxin GVIA blocked approximately 85% of the remaining calcium current with an IC_{50} of 3 nM and a Hill coefficient of 1. The block by conotoxin GVIA was irreversible on the time scale of these experiments. These results suggested that the majority of the nifedipine-insensitive calcium current was N-type.

3 Fluspirilene, a substituted diphenylbutylpiperidine with potent neuroleptic properties, reversibly inhibited the N-type component in a dose-dependent manner with an IC_{50} of 30 nM. The Hill coefficient of the block was 0.25. The fraction of current blocked was the same at all test potentials examined (−30 to +40 mV).

4 These data indicate that the neuroleptic properties of fluspirilene may be due, at least in part, to an inhibition of neuronal N-type calcium channels. This finding raises the possibility that modulation of N-type calcium channel activity by drugs derived from substituted diphenylbutylpiperidines may provide a novel way of altering neurotransmitter release and hence brain function.

Keywords: Diphenylbutylpiperidine; calcium channels; calcium channel antagonists; neuroleptic agents; N-type calcium channels; PC12 cells

Introduction

Fluspirilene, a member of the diphenylbutylpiperidine (DPBP) class of neuroleptic drugs (which also includes pimozide, clopimozide and penfluridol), has been shown to have anti-schizophrenic actions (Hassel, 1985). The first evidence for the mechanism of action of DPBPs was the observation that fluspirilene and other DPBPs inhibit [^{3}H]-nitrendipine binding to rat cortical membrane vesicles (Gould *et al.*, 1983). Subsequent binding studies on skeletal muscle (Galizzi *et al.*, 1986), cardiac muscle (Quirion *et al.*, 1985; Qar *et al.*, 1987; King *et al.*, 1989), smooth muscle (Qar *et al.*, 1987) and brain (Quirion *et al.*, 1985; Qar *et al.*, 1987; Kenny *et al.*, 1990) showed that DPBPs bind to receptors for calcium channel antagonists in a variety of tissues. However, the binding site for DPBPs appears to be a discrete site, separate from the dihydropyridine, arylalkylamine and benzothiazepine binding sites of the calcium channel (King *et al.*, 1989).

Fluspirilene, the DPBP with the highest affinity in binding assays, has been shown to block calcium channels in functional studies. Inhibition of calcium movements has been demonstrated in both smooth muscle (Gould *et al.*, 1983; Fraser *et al.*, 1988; Kenny *et al.*, 1990) and neuronal (Galizzi *et al.*, 1986; Qar *et al.*, 1987; King *et al.*, 1989; Enyeart *et al.*, 1990) preparations. Whole-cell patch clamp data have shown that fluspirilene blocks both low voltage-activated (T-type) and high voltage-activated calcium channels in GH3 and GH4C1 cell lines (Enyeart *et al.*, 1990).

Nowycky *et al.* (1985), working with chick dorsal root ganglion cells, introduced a classification for high-voltage-activated (HVA) calcium channels, dividing them into L- and N-type channels. N-type channels are found predominantly in neurones and differ from L-type channels in both their electrophysiological and pharmacological properties (Fox *et al.*, 1987). The CNS distribution of these N-type channels is widespread and is distinct from that of L-type channels (Sher & Clementi, 1991). At the cellular level, N-type channels are clustered at 'active zones' on presynaptic nerve terminals (Smith & Augustine, 1988) and have been implicated in the control of neurotransmitter release from these structures (Hirning *et al.*, 1988).

Previous mechanistic studies did not differentiate between N-type and L-type calcium channels, so it is possible that the anti-schizophrenic action of fluspirilene may be due, at least in part, to modulation of neurotransmitter release via an effect on central N-type channels. To examine this possibility, we used a nerve growth factor (NGF)-differentiated PC12 cell line as a source of N-type calcium channels. PC12 cells are derived from a rat phaeochromocytoma cell line and respond to NGF by acquiring neuronal characteristics, such as extension of neurite-like processes, and by expression of voltage-sensitive sodium and calcium channels (Streit & Lux, 1987; Rudy *et al.*, 1987; Furukawa *et al.*, 1993). A number of studies have demonstrated that the calcium channels in NGF-differentiated PC12 cells can be sub-divided into the types described by Nowycky *et al.* (1985), namely, L-, N- and T-type channels (Garber *et al.*, 1989; Usowicz *et al.*, 1990; Plummer *et al.*, 1989). In this study, we have shown that our line of NGF-differentiated PC12 cells expresses N-type calcium channels and that fluspirilene blocks calcium fluxes through these channels. We conclude, therefore, that a part of the central action of fluspirilene may be the result of an effect on central neurotransmitter release through its antagonism of N-type calcium channel fluxes.

Methods

Growth and preparation of differentiated PC12 cells

PC12 cells, adherent to tissue culture flasks, were isolated by agitation without enzymes, from maintenance cultures which

[1] Author for correspondence.

were routinely passaged once weekly. This procedure produced large clumps of cells which could be dispersed by gentle trituration through a 21-gauge needle up to 5 times to generate a largely unicellular suspension. Induction of neuronal phenotype was achieved by transferring the dispersed cells to poly-L-lysine/collagen-coated glass coverslips and exposing them to NGF in defined culture medium at 37°C in a humidified 95% air/5% CO_2 atmosphere (Doherty *et al.*, 1988). Under this differentiating protocol the PC12 cells' appearance changed from relatively indistinct, masses of dividing cells to one of single, non-dividing, refractile, spherical cells, each developing one or more processes after 1 to 2 days exposure to NGF.

Recording of N-type calcium currents by whole-cell patch clamp technique

Coverslip fragments bearing adherent, differentiated PC12 cells were transferred to a recording chamber, mounted on the stage of a Nikon Diaphot microscope (Nikon Instruments, Japan). The chamber (volume 200 µl) was continuously perfused with either Ca/Na EBS or Ca/TEA EBS flowing at a rate of 2 ml min^{-1}. Drugs were added to the Ca/TEA EBS as required. Low resistance patch electrodes (2–4 MOhm) were pulled from fibre-filled borosilicate glass (Sutter Instruments, U.S.A.) and were filled with a cesium containing solution (see below) to suppress potassium currents.

The whole-cell patch-clamp technique (Hamill *et al.*, 1981) was used to examine voltage-sensitive calcium channels (VSCCs) in NGF-treated PC12 cells. Cells, bathed in Ca/Na EBS, were voltage-clamped with an Axopatch 1B patch clamp amplifier (Axon Instruments Inc., U.S.A.). After achieving whole-cell configuration, the bathing solution was switched to Ca/TEA EBS, to suppress potassium and sodium currents. The PC12 cell VSCCs were examined using stimulation protocols generated by pCLAMP version 5 driving a TL-1 analogue-to-digital converter (Axon Instruments Inc., U.S.A.) in a 486/25 MHz microcomputer (Gateway, U.S.A.). Data were filtered at 5 kHz and digitized by a VR-4-100A digital recorder (Instrutech, U.S.A.) for recording by a VCR. Data were analysed off-line with pCLAMP software.

A ramp protocol was used to obtain a rapid confirmation of the presence of calcium currents in individual cells. The cells were then stepped to the potential which evoked a peak current in order to characterize the whole-cell PC12 current in terms of nifedipine and ω-conotoxin GVIA (CgTX) sensitive components.

Solutions (composition in mM)

Ca/Na EBS $CaCl_2$ 2, KCl 5.4, NaCl 135, $MgCl_2$ 1, (N-[2-hydroxyethyl]piperizine-*N'*-[2-ethanesulphonic acid]) (HEPES) 10, glucose 10, pH 7.4 (adjusted with NaOH).

Ca/TEA EBS $CaCl_2$ 10, KCl 5.4, NaCl 135, $MgCl_2$ 1, TEA Cl 20, HEPES 10, glucose 10, tetrodotoxin 300 nM, pH 7.4.

Pipette solution CsCl 10, CsOH 100, aspartic acid 80, TEA Cl 20, HEPES 30, EGTA 5, MgATP 3, pH 7.2 (adjusted with CsOH).

Culture medium Growth medium for PC12 cells was Dulbecco's Modified Eagle's Medium (Imperial Laboratories, Andover, U.K.) supplemented with 2% foetal calf serum (Sera Lab, U.K.), glutamine 2 mM, penicillin 50 u ml^{-1}, streptomycin 50 µg ml^{-1} (Life Technologies Ltd., Paisley, U.K.), bovine insulin 0.6 iu ml^{-1}, human apo-transferrin 0.125 µg ml^{-1} (Sigma Chemical Co., Poole, U.K.) and SATO's ingredient's (sodium selenite 0.3 µM, progesterone 0.25 µM, L-thyroxine 0.6 µM, triiodothyronine 0.6 µM, putrescine 125 µM, Path-O-cyte-4 1%; Sigma and ICN Biomedicals Ltd., High Wycombe, U.K.) as described by Doherty *et al.* (1988).

Differentiating medium was culture medium without the addition of calf serum and included 100 ng ml^{-1} 7S-nerve growth factor (Calbiochem Novabiochem, Nottingham, U.K.).

Chemicals and materials

All tissue culture-ware was obtained from BD U.K. (Oxford, U.K.), coverslips (No. 0, Chance Proper, Warley, U.K.) were first coated with aqueous poly-L-lysine (Sigma) 50 µg ml^{-1} followed by collagen 25 µg cm^{-2} in 30/20 (v/v) ethanol/water mixture (rat-tail type-VII, Sigma) and air dried before use. Cadmium chloride (Sigma) and CgTX (Bachem, California, U.S.A.) stocks were made up in aqueous solution, while dimethylsulphoxide (DMSO) (Sigma) was the vehicle for nifedipine (Sigma) and fluspirilene (synthesized at Lilly Research Centre Ltd., by J. Fairhurst) stocks. All other chemicals were of reagent grade (Sigma; Fisons, Loughborough, U.K.; Aldrich, Gillingham, U.K.) and all solutions were made up in purified water (>18 MOhm cm^{-1}).

Results

Undifferentiated cells and cells exposed to NGF for less than 3 days had little or no high voltage-activated (HVA) calcium currents. Exposure of cells to NGF for between 3 and 10 days resulted in the growth of neurites and concomitant appearance of HVA calcium current. Cells with only one or two short neurites were selected for experiments as it was found impossible to obtain adequate voltage control in cells with long multiple neurites. Longer term exposure to NGF resulted in a reduction in the number of cells in which it was possible to obtain adequate voltage control (not shown). In chosen cells, the whole-cell calcium current ranged from 200 pA to 1 nA.

Kinetics and cadmium-sensitivity of PC12 calcium current

As illustrated in Figure 1, the bathing and pipette filling solutions allowed measurement of calcium currents without serious contamination from other conductances. Figure 1a shows whole-cell currents generated in response to a depolarizing voltage ramp from −100 mV to +100 mV over 500 ms (holding potential = −40 mV). At very negative potentials the current voltage relationship is linear with a slope conductance of 0.4 pS. This low conductance probably reflects the integrity of the seal between the membrane and pipette (as well as any residual membrane conductance). As the membrane potential became more positive than about −10 mV, a large inward current was observed which reached a peak of 275 pA at +20 mV before decreasing and reversing near to +60 mV. The addition of 0.1 mM Cd^{2+} blocked the inward current, suggesting that the recorded current was due to the activation of HVA calcium currents.

Characterization of nifedipine-resistant calcium current in NGF-treated PC12 cells

Since there are at least three types of HVA calcium channel in neuronal preparations (Nowycky *et al.*, 1985), pharmacological criteria were used to distinguish between the types of calcium channel present. As illustrated in Figure 1b, depolarization beyond −20 mV elicited a rapidly activating inward current which slowly inactivated during the pulse. Addition of 10 µM nifedipine (a dose that produces maximal block of L-type calcium channels: Nowycky *et al.*, 1985) reduced the current elicited in response to depolarizing steps by about 50% at all potentials examined. Subsequent exposure of the cell to 100 nM CgTX resulted in a further

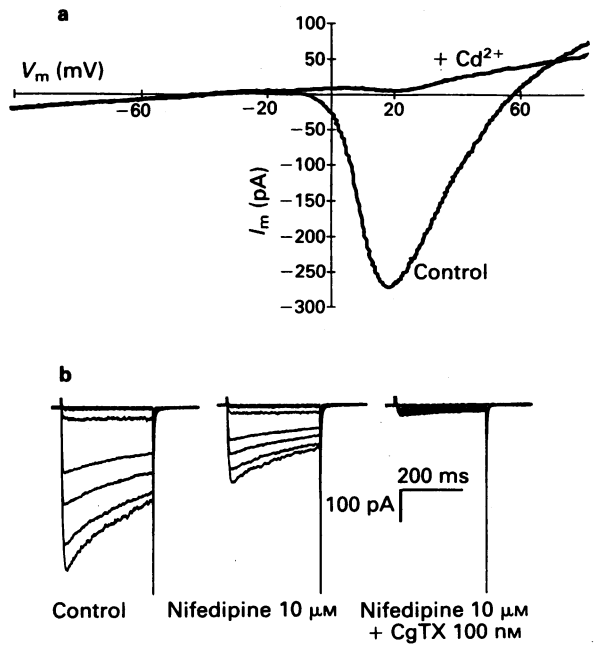

Figure 1 Characterization of PC12 cell calcium current and its sensitivity to inorganic and organic agent(s). (a) Whole-cell recording from a nerve growth factor (NGF)-treated PC12 cell showing the current-voltage relationship before and after cadmium chloride (0.1 mM). The calcium current activated at −10 mV, peaked at +20 mV and reversed at about 60 mV, which was the reversal potential for Ca^{2+} under the conditions used in this series of experiments. Cadmium almost completely blocked the current within 30 s of application and could be washed off with similar rapidity (not shown). (b) Typical PC12 calcium currents evoked by steps from the holding potential (−40 mV) to various potentials between −20 mV and 60 mV in 10 mV increments. The step duration was 300 ms and steps were applied at 5 s intervals. The current present in control and nifedipine-treated traces contained a large component of time-dependent inactivation which largely disappeared after treatment with ω-conotoxin GVIA (CgTX). Application of 10 μM nifedipine resulted in approximately a 50% reduction in current at all voltages without affecting the kinetics of current activation. Application of 100 nM CgTX caused a further reduction of current at all voltages to about 10% of the control current. Note the residual current remaining after combined application of both nifedipine and CgTX.

reduction in the amplitude of the calcium current to 12% of the control. It is possible that this residual current reflects the presence of P-type calcium channels (Llinas *et al.*, 1989; Mintz *et al.*, 1992), but this idea was not explored further. Experiments of the type shown in Figure 1 showed that NGF exposure resulted in the expression of a HVA-calcium current which consisted predominantly of L- and N-type current in approximately equal proportions.

The time course of the block of nifedipine-insensitive current is illustrated in Figure 2a. The cell had been exposed to nifedipine (10 μM) until the current reached a steady level before CgTX was added. CgTX exposure caused the current to decrease with a half time of about 60 s to a new steady level. The dose-response relationship for the inhibition of the nifedipine-insensitive current by CgTX is shown in Figure 2b. Regression analysis suggested that the block of the nifedipine-insensitive current by CgTX was dose-dependent with half maximal block occurring at 3 nM and a Hill coefficient of 1. It is possible that channel run-down contributed to the observed block at 1 nM CgTX since the development of block at this CgTX concentration was very slow ($T_{1/2} > 5$ min). The block by CgTX was not reversible on the time scale of these experiments (not shown). As noted earlier, a component of PC12 calcium current was not blocked by the combination of nifedipine and CgTX, and in these experiments this resistant component comprised about 25% of the nifedipine-resistant current. The inset to Figure 2a shows sample records from an experiment performed to illustrate the graded block of current by CgTX. Three doses (1, 30 and 100 nM) of CgTX were applied sequentially to a single cell. The currents were elicited by steps from a V_{hold} of −40 mV to +20 mV for 200 ms every 5 s.

Effect of fluspirilene on the nifedipine-resistant calcium current in PC12 cells

The above data show that after NGF exposure, the PC12 cells expressed both N- and L-type calcium channels. Since fluspirilene blocks L-type channels (Qar *et al.*, 1987; King *et al.*, 1989; Enyeart *et al.*, 1990), the contribution of L-type channels to the whole-cell current was removed by applying 10 μM nifedipine. As the majority of the residual current in these conditions (about 80%) was due to N-type calcium channels (as shown by its sensitivity to CgTX), it was possible to examine the block of N-type calcium current by fluspirilene. Figure 3a shows that application of 10 μM nifedipine blocked about 50% of the whole-cell current in about 1 min. After the block by nifedipine had stabilized, 10 μM fluspirilene was added to the bathing solution resulting in a further decrease in the amplitude of the calcium current.

Figure 3b shows the dose-dependence of the block by fluspirilene. Fluspirilene reduced the nifedipine-resistant cur-

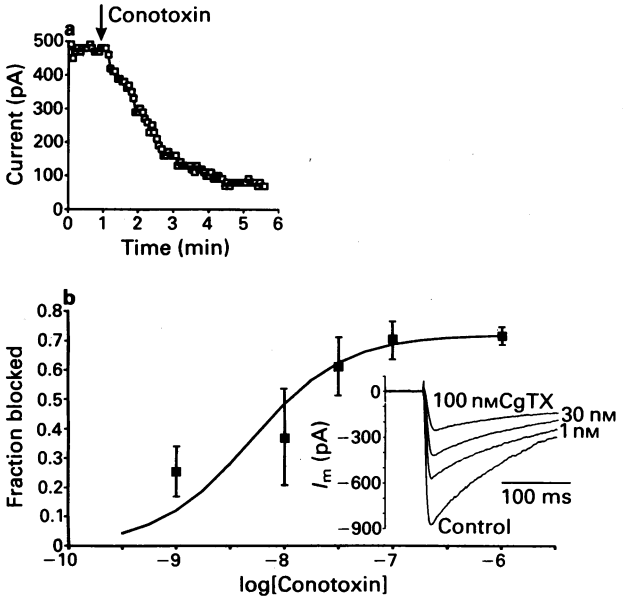

Figure 2 Time course and sensitivity of PC12 cell calcium current to ω-conotoxin GVIA (CgTX) in the presence of nifedipine (10 μM). (a) The time course of inhibition of calcium current after exposure to 100 nM CgTX illustrates a relatively stable current before and after CgTX, with 50% inhibition occurring about 150 s after CgTX application began. Note that a residual current remained after CgTX application amounting to about 20% of the nifedipine-resistant current. A ten fold increase in the dose of CgTX (to 1 μM) did not affect this residual current (data not shown) suggesting that 100 nM CgTX exerted a maximal effect in this preparation. (b) The main panel shows the dose-response to CgTX in the presence of nifedipine (10 μM). Each point shows the mean and standard deviation of pooled data from separate experiments on different cells ($n = 2-5$). The solid line shows a least squares fit to the Hill equation giving an IC_{50} of 3 nM and a Hill coefficient of 1, suggesting that the stoichiometry of inhibition was one CgTX molecule per calcium channel. However, the mean ± s.d. for 1 nM CgTX fell above this curve, possibly due to channel run-down during the long period of time required to attain steady-state at this concentration. The inset shows sample records from an experiment in which three doses (1, 30 and 100 nM) of CgTX were applied sequentially to a single cell. The currents were elicited by steps from a V_{hold} of −40 mV to +20 mV for 200 ms every 5 s.

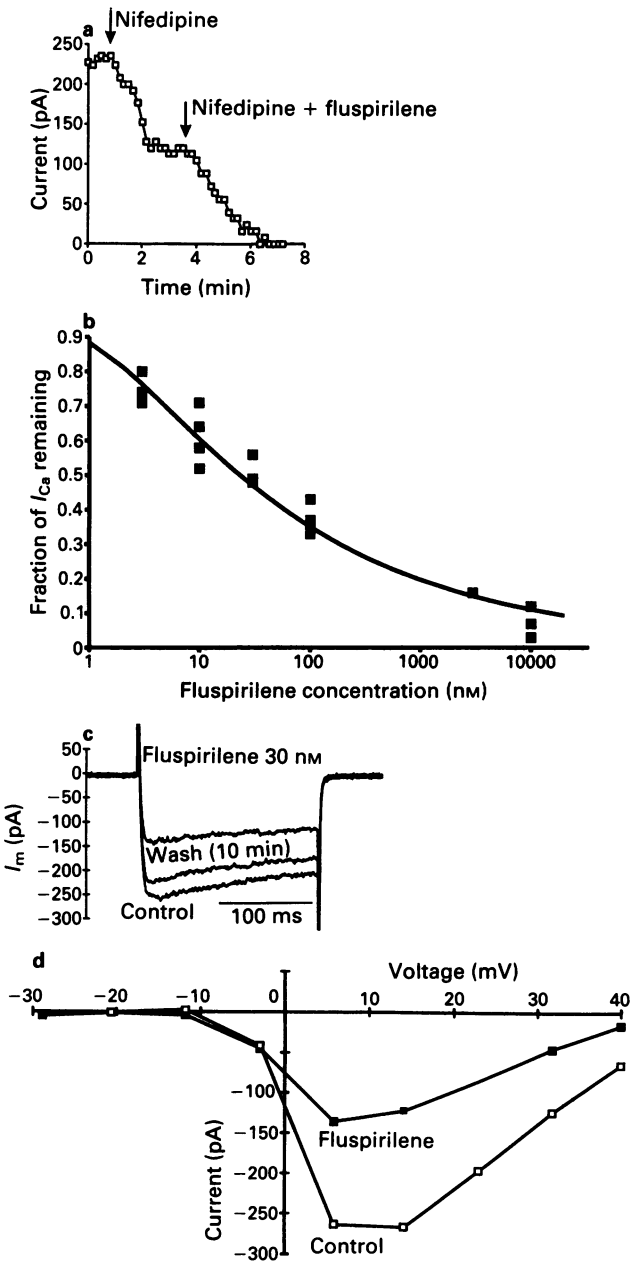

Figure 3 Sensitivity and current-voltage relationship of ω-conotoxin GVIA (CgTX)-sensitive PC12 calcium current to fluspirilene. (a) The time course of current inhibition shows that about 50% of the whole-cell current remained after application of nifedipine (10 μM) and that the $t_{1/2}$ of nifedipine action was about 30 s. After a steady state was reached, fluspirilene (10 μM) was added to the bathing solution and in this cell caused complete abolition of current within 2.5 min. (b) The dose-response curve for fluspirilene was constructed from peak currents measured in the presence of nifedipine (10 μM) in different cells at various doses of fluspirilene using a similar protocol to the one used to generate the dose-response to CgTX. The curve, which shows the individual data points, is shallow with half maximal inhibition of calcium current at about 30 nM fluspirilene. The data were fitted by a Hill equation which gave a Hill coefficient of 0.25, suggesting that the block induced by fluspirilene is more complicated than that produced by CgTX. Note that at 10 μM fluspirilene there was some residual current (about 10% of the nifedipine-resistant current). (c) The reversibility of fluspirilene was investigated with a single step pulse of 200 ms duration to 15 mV from a V_{hold} of −40 mV. A peak current of 260 pA was evoked under this protocol in the presence of nifedipine (10 μM). As shown, fluspirilene (30 nM) reduced the nifedipine-resistant current by about 50%. After washing for 10 min the current recovered to about 85% of its pre-fluspirilene value, indicating that fluspirilene-induced blockade was reversible. The 15% difference between pre- and post-fluspirilene current may be ascribed to current run-down during the 10 min wash period. (d) This panel shows the voltage-dependence of the calcium current in the presence of nifedipine (10 μM) and after the addition of fluspirilene (30 nM). The holding potential was −40 mV and the step duration was 200 ms. The nifedipine-insensitive current activated at about −10 mV and peaked at about 10 mV. The addition of 30 nM fluspirilene reduced the current by approximately 50% at all potentials without any shift in the *I-V* relationship.

rent in a dose-dependent manner with half maximal block occurring at 30 nM. However, the block of the nifedipine-insensitive current by fluspirilene was more complex than that due to CgTX, since the Hill coefficient of the curve fitted to the data was only 0.25. It is notable that Hill coefficients of less than unity have also been reported in binding studies investigating displacement of PN-200-110 by fluspirilene (Kenny *et al.*, 1990) as well as fluspirilene displacement by nitrendipine in rat cardiac sarcolemmal membrane vesicles (King *et al.*, 1989). The fraction of current blocked by fluspirilene did not increase during the test pulse. In four experiments 1 μM fluspirilene blocked 67 ± 8% of the peak and 74 ± 11% of the steady state nifedipine-insensitive current.

Figure 3c shows that the block of nifedipine-insensitive current by fluspirilene was reversible. As shown earlier, application of 30 nM fluspirilene blocked approximately 50% of the nifedipine-resistant current; 10 min after washing out the fluspirilene, the evoked calcium current returned to 85% of its control value. Although recovery from fluspirilene block was incomplete in these experiments, it is likely that calcium current run down could account for the deficit.

Current-voltage relationship of fluspirilene action on the PC12 nifedipine-resistant calcium current

To examine whether the observed block of N-type current by fluspirilene was due to an alteration of channel gating, the current-voltage relationship was measured by applying pulses to different potentials. As shown in Figure 3d, the current activated at about −10 mV and reached a peak at about +10 mV and then decreased again as the reversal potential of the calcium current was approached. The addition of 30 nM fluspirilene decreased the amplitude of the current by about 50% at all potentials examined, suggesting that the block by fluspirilene was not due to a shift in the voltage-dependence of calcium current gating.

Discussion

It is clear that multiple subtypes of voltage-sensitive calcium channels (VSCC) are present in neuronal tissues (Nowycky *et al.*, 1985). In addition, at the level of the single neurone, co-expression of different subtypes of VSCC may occur (Miller, 1987). A number of groups have used the NGF-differentiated PC12 cell-line as a model for examining neuronal calcium channel properties (Plummer *et al.*, 1989; Streit & Lux, 1989; 1990; Usowicz *et al.*, 1990). In all studies, a HVA calcium current was expressed but was not generally subdivided into L- and N-type components. Both electrophysiological and pharmacological criteria have been used to characterize HVA VSCCs (Fox *et al.*, 1987). Dihydropyridine antagonists such as nifedipine and nitrendipine have been shown to block selectively L-type calcium channels (e.g. Nowycky *et al.*, 1985). In our experiments, nifedipine blocked a substantial proportion of the whole-cell calcium current suggesting that there is an L-type component to the HVA current exhibited by PC12 cells. This result is in agreement with the results of Usowicz *et al.* (1990) and Plummer *et al.* (1989).

CgTX, a toxin originally isolated from the venom of the piscivorous cone snail, *Conus geographcus* (Olivera *et al.*, 1984), has been widely used in the identification of N-type

HVA channels (Sher & Clementi, 1991). Usowicz *et al.* (1990) and Plummer *et al.* (1989) demonstrated that in the NGF-differentiated PC12 cell line, CgTX is selective for the N-type channel. In our experiments CgTX blocked a large proportion of the DHP-insensitive current suggesting that L-type and N-type calcium channels are the major channel types expressed in the NGF-treated cells.

As mentioned previously, a small component of the whole-cell current was found to be resistant to blockade by high concentrations of nifedipine and CgTX in combination. Several other types of HVA VSCC, showing resistance to CgTX and nifedipine have been identified recently (P-type – Llinas *et al.*, 1989; Rabbit brain B1 - Mori *et al.*, 1991). The P-type channel, first identified in rat Purkinje neurones and described by Llinas *et al.* (1989), is blocked by AgaIVA, a funnel web spider toxin but not by CgTX (Mintz *et al.*, 1992). It is possible that our residual calcium current represents such a P-type channel. However, further experiments with AgaIVA would be necessary to examine this point.

Fluspirilene is known to interact with HVA calcium channels in skeletal muscle (Galizzi *et al.*, 1986), brain, cardiac and smooth muscle (Quirion *et al.*, 1985; Qar *et al.*, 1987; King *et al.*, 1989; Kenny *et al.*, 1990). In previous studies, the receptor protein was only probed with classical L-type calcium channel antagonists (such as dihydropyridines and phenylaklylamines). Thus, the inhibition of N-type calcium channels by fluspirilene observed here is a novel result.

As noted previously, fluspirilene exhibits a low Hill coefficient for dihydropyridine displacement in binding studies (Kenny *et al.*, 1990). It is notable that we observed a similar low Hill coefficient for the block of N-type (CgTX-sensitive, nifedipine-insensitive) current. However, the low Hill coefficient for fluspirilene inhibition is not easily explainable on the basis of a displacement of nifedipine (and hence a relief of nifedipine block) by fluspirilene as fluspirilene blocks and binds to L-type channels with an affinity of about 22 nM (Gould *et al.*, 1983) which is very close to that observed for N-type channels in these experiments.

An alternative explanation might be that the affinity of the N-type channel for fluspirilene depends on membrane potential (as reported for L-type channels by Enyeart *et al.*, 1990) so that complete blockade of channels examined with a pulse protocol would not occur until the low affinity state was completely occupied by fluspirilene. This model could give a more shallow dose-response than would be seen if there were no change in affinity with depolarization. However, the fraction of current blocked was not sensitive to test pulse potential in the range examined (Figure 3d) so that the low Hill coefficient is not easily explained by the production of a higher affinity state during the test pulse. Furthermore, the fraction of current blocked did not increase during the pulse (see also Figure 3c) so that we have no evidence for the affinity of the channel changing during depolarization. A related explanation for the low Hill coefficient could be that there is more than one binding site for fluspirilene, and each site produces partial blockade with different affinities. Such a model will produce inflexions in the binding curve, that may only be detected if the affinities of the binding sites are sufficiently different.

As a neuroleptic drug, fluspirilene has been used clinically to treat schizophrenic symptoms for many years. Fluspirilene and a few other DPBPs like pimozide and penfluridol are unique among neuroleptic drugs in that they alleviate both the 'positive' and 'negative' symptoms of schizophrenia (Lapierre, 1978; Haas & Beckmann, 1982). Neuroleptic effects against the 'positive' manifestations such as hallucinations and delusions have commonly been ascribed to dopamine antagonism at the D_2 receptor (Seeman, 1980). Yet, in contrast to classical non-DPBP neuroleptics such as haloperidol, fluspirilene also relieves emotional withdrawal symptoms (a 'negative' characteristic of schizophrenia) and additionally instils increased self-confidence together with relaxation (Hassel, 1985), which are features of an anxiolytic agent. These additional properties may be the result of an action of fluspirilene on synaptic transmission not shared by other classical neuroleptic agents. The finding that fluspirilene also blocks N-type calcium channels may therefore provide an explanation for the additional anxiolytic effects of fluspirilene. In support of this idea is the clinical observation that the anxiolytic property of fluspirilene occurs at very low dose levels (Hassel, 1985), a finding which parallels the high potency of this drug on N-type calcium channels reported here. Since N-type channels are believed to be concentrated at sites of synaptic transmission, a N-type channel antagonist which crosses the blood-brain barrier would be expected to be a potent modifier of brain function.

Although not selective for N-type calcium channels, fluspirilene may potentially have a clinical role as a potent, brain penetrable calcium antagonist which could be used to treat disorders resulting from abnormal neuronal cell depolarization. In connection with this point, it is notable that Gandolfo *et al.* (1989), using a rat model of epilepsy, found that fluspirilene potently blocks epileptiform activity via a suppression of neuronal hyperexcitability. The fact that fluspirilene blocks both N- and L-type calcium channels may indicate that there is a region of structural homology between these channel types. It is also possible that chemical modification of the fluspirilene molecule may confer differential selectivity between HVA calcium channel subtypes. Should this be the case, derivatives of fluspirilene could provide novel therapeutic agents for treating disorders of the central nervous system.

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