Selective inhibition of high voltage-activated L-type and Q-type Ca^{2+} currents by serotonin in rat melanotrophs

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1. Whole-cell Ca^{2+} currents (I_{Ca}) from cultured rat melanotrophs were identified by their sensitivity to Ca^{2+} channel blockers, and their modulation by serotonin (5-HT) was studied. All cells displayed high voltage-activated (HVA; >-30 mV) Ca^{2+} currents. A low voltage-activated (LVA; >-60 mV) Ca^{2+} current was detected in 92% of the cells.

2. The whole-cell I_{Ca} was insensitive to ω-conotoxin GVIA (0·5–1 μM) indicating the absence of N-type Ca^{2+} channels.

3. At a holding potential (V_h) of -70 mV, the L-type channel blocker nifedipine reduced I_{Ca} in a dose-dependent manner with a half-maximal effective concentration (IC_{50}) of 28 nM. The L-type current represented 39% of the total I_{Ca}.

4. ω-Agatoxin IVA (ω-Aga IVA) produced a biphasic dose-dependent inhibition of I_{Ca}, with IC_{50} values of 0·4 and 91 nM, indicating the presence of P-type and Q-type Ca^{2+} channels, which accounted respectively for 16 and 45% of the total I_{Ca}. The P-type current was also blocked by synthetic funnel-web spider toxin (sFTX 3.3; 1–10 μM) and was present only in a subpopulation (60–70%) of cells.

5. All cells possessed a Ca^{2+} current which was resistant to nifedipine (10 μM) and ω-Aga IVA (50 nM). This current was not affected by Ni^{2+} (40 μM) but was abolished by a low concentration of Cd^{2+} (10 μM) and by ω-conotoxin MVIIC (1 μM) indicating that it was a Q-type Ca^{2+} current.

6. 5-HT (10 μM) inhibited the whole-cell I_{Ca} in 70% of the cells tested ($n = 120$) by activating 5-HT_{1A} and 5-HT_{2C} receptors. 5-HT produced either a kinetic slowing of the activation phase (37% of the cells) or a scaling down (14% of the cells) of I_{Ca}. In the majority of cells (49%) both types of inhibition were found to coexist.

7. The effects of 5-HT were voltage dependent, rendered irreversible when GTP-γ-S (30 μM) was present in the pipette solution and abolished by pretreatment of the cells with pertussis toxin (PTX; 150 ng ml^{-1}, 18 h).

8. Low concentrations of ω-Aga IVA (20 nM), which blocked mainly P-type channels, did not reduce the effect of 5-HT on I_{Ca}. The scaling down effect of 5-HT on I_{Ca} was eliminated in the presence of nifedipine (10 μM) and the kinetic slowing effect of 5-HT persisted after blockade of L- and P-type channels but was abolished by ω-conotoxin MVIIC (1 μM).

9. We conclude that rat melanotrophs possess functional L-, P- and Q-type Ca^{2+} channels and that 5-HT inhibits selectively L-type and Q-type Ca^{2+} currents with different modalities. These effects are voltage dependent and mediated by a PTX-sensitive G-protein.

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The endocrine cells of the intermediate lobe of the pituitary (melanotrophs) constitute a homogeneous population of excitable cells which share many properties of neurones. For example, they possess low voltage-activated (LVA) and high voltage-activated (HVA) Ca^{2+} currents which underlie the generation of Ca^{2+}-dependent action potentials (Williams, MacVicar & Pittman, 1990*a*). These Ca^{2+} currents in turn mediate Ca^{2+} influx, which is directly related to the secretion of α-melanocyte-stimulating hormone (α-MSH; Nemeth, Taraskevich & Douglas, 1990). The secretion of this hormone is modulated by neurotransmitters such as dopamine, which is known to inhibit Ca^{2+} currents in melanotrophs when released synaptically after electrical stimulation of the pituitary stalk (Williams, MacVicar & Pittman, 1990*b*). The intermediate lobe of the pituitary is also innervated by nerve fibres containing serotonin (5-HT; Mezey, Leranth, Brownstein, Friedman, Krieger & Palkovits, 1984) and we have previously shown that porcine melanotrophs possess functional 5-HT receptors mediating the inhibition of a component of the HVA Ca^{2+} current (Ciranna, Mouginot, Feltz & Schlichter, 1993). An inhibitory effect of 5-HT on HVA Ca^{2+} currents has also been described in acutely dissociated rat dorsal raphe neurones (Penington, Kelly & Fox, 1991). In both preparations, however, an important issue is to determine the nature of the Ca^{2+} current modulated by 5-HT. This is particularly true in light of the recent finding that at least five different subtypes of HVA Ca^{2+} channels can coexist in the membrane of the same neurone (Zhang *et al.* 1993; Randall & Tsien, 1995). The distinction between these Ca^{2+} current subtypes is essentially based on their sensitivity to pharmacological blocking agents. For example, it is well documented that L-type Ca^{2+} currents are blocked by 1,4-dihydropyridines (Scott, Pearson & Dolphin, 1991). However, until recently, blocking agents allowing a clear separation of the other components of the HVA Ca^{2+} current were not available. To date, the distinction between these different subtypes of Ca^{2+} channels is facilitated by the use of toxins isolated from marine snail or spider venoms. Thus N-type channels are blocked by ω-conotoxin GVIA (ω-CTX GVIA; McCleskey *et al.* 1987; Plummer, Logothetis & Hess, 1989; Kasai & Neher, 1992) whereas P-type channels are inhibited by funnel-web spider toxin (FTX; Llinás, Sugimori, Hillman & Cherksey, 1992) and by low nanomolar concentrations of ω-agatoxin IVA (ω-Aga IVA; Mintz, Venema, Swiderek, Lee, Bean & Adams, 1992; Randall & Tsien, 1995). Moreover, two novel types of Ca^{2+} currents termed Q-type and R-type have been identified in cerebellar granule cells (Zhang *et al.* 1993; Randall & Tsien, 1995). The R-type current is resistant to dihydropyridines and to all known toxins blocking Ca^{2+} channels (Randall & Tsien, 1995). In contrast, the Q-type current is blocked by high concentrations of ω-Aga IVA (Randall & Tsien, 1995) and by ω-CTX MVIIC, which also inhibits N- and P-type Ca^{2+} currents (Hillyard *et al.* 1992; Randall & Tsien, 1995). In view of the diversity of Ca^{2+} channels existing in neurones we have decided to re-examine the pharmacological properties of Ca^{2+} currents present in melanotrophs in order (i) to determine the nature of the Ca^{2+} current subtypes underlying the HVA Ca^{2+} current of these endocrine cells and (ii) to study their modulation by 5-HT. Our results show that melanotrophs possess functional L-, P- and Q-type Ca^{2+} channels and that 5-HT inhibits the HVA component of the whole-cell Ca^{2+} current by inducing a scaling down of the L-type current and a kinetic slowing of the Q-type current, while leaving the P-type current unaffected.

METHODS

Tissue culture

Pituitary glands were removed from 7-day-old rats after decapitation under deep diethyl ether anaesthesia, and collected in phosphate buffer solution (PBS; containing (mM): $CaCl_2$, 0·4; $MgCl_2$, 0·24; KCl, 1·35; KH_2PO_4, 0·73; NaCl, 68·9; and Na_2HPO_4, 1·6). Neurointermediate lobes (NILs) were separated from anterior lobes under a stereomicroscope using thin forceps. The NILs were then transferred to a dissociation medium composed of a Ca^{2+}- and Mg^{2+}-free PBS to which were added 5 mg ml^{-1} dispase (neutral protease, grade II, Boehringer) and 1 mg ml^{-1} collagenase (clostridiopeptidase A, Type IV, Sigma). After 15 min of incubation at 37 °C, the fragments of tissue were washed with culture medium composed of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated horse serum (Gibco), and triturated with a fire-polished Pasteur pipette. The dissociated cells were plated on poly-L-ornithine-coated Costar dishes (poly-L-ornithine hydrobromide, Sigma, 10 μg ml^{-1}), and kept at 37 °C in a 95% air and 5% CO_2 humidified atmosphere, until use in electrophysiological experiments (3–7 days after plating).

Electrophysiological recordings

Voltage-activated calcium currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), using a List EPC7 amplifier. Experiments were performed at room temperature (22–25 °C). Electrodes were pulled with a vertical puller (L/M-3P-A; List) using haematocrit glass tubing (Wu, Mainz, Germany). Pipette tip resistances ranged from 2 to 4 MΩ, when filled with intracellular solution. Electrophysiological data were stored on the hard disk of an IBM-compatible computer (Olivetti M290) and analysed off-line with the pCLAMP software (Axon Instruments). During the experiments, current traces were continuously monitored on a digital oscilloscope (Tektronix 2220) and on a chart recorder (Gould 2200S). Leak currents were subtracted on-line from the test current trace using a *P* over *N* protocol (*P*/*N*, pCLAMP software); each test pulse was preceded by a number *N* (5 or 6) of subpulses, the amplitude of which corresponded to the test pulse amplitude divided by the number of subpulses. The amplitude of each subpulse was too small to elicit active currents.

Solutions for electrophysiological recordings

Calcium currents were isolated by blocking all other membrane currents by ionic substitution and pharmacological agents.

Extracellular sodium and intracellular potassium were replaced by *N*-methyl-D-glucamine (NMG) and tetraethylammonium (TEA; 20 mM) was added to the extracellular solution in order to achieve complete blockade of potassium currents. The composition of the

standard extracellular solution was the following (mM): NMGCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; TEACl, 20; Hepes, 5; glucose, 10; pH 7·3. Electrodes were filled with intracellular solution containing (mM): NMGCl, 140; $MgCl_2$, 2; Hepes, 10; $CaCl_2$, 5; EGTA, 10; Mg-ATP, 2; Na-GTP, 0·3; pH 7·3. Under these conditions, the final intracellular free calcium concentration was 0·1 μM. ATP was added to minimize the run-down of Ca^{2+} currents.

During the experiments, the extracellular solution contained in the perfusion chamber (either 300 μl or 2 ml) was continuously changed by a gravity-driven bath-perfusion system, at a rate of 2 ml min^{-1}.

Drugs and chemicals

Drugs were applied either by bath perfusion or locally using a U-tube (Fenwick, Marty & Neher, 1982). All substances were prepared as intermediate stock solutions and diluted to the desired final concentration in extracellular solution just before the experiment. Nifedipine was prepared as a 10 mM stock solution in dimethyl sulphoxide (DMSO). ω-CTX GVIA, ω-CTX MVIIC and ω-Aga IVA were prepared as stock solutions in extracellular medium at concentrations of 10 mM, 10 μM and 1 μM, respectively, and then diluted to the final concentration in extracellular medium. Cytochrome *c* (0·1 mg ml^{-1}) was added to both stock and final solutions in order to prevent non-specific peptide binding to containers (Sather, Tanabe, Zhang, Mori, Adams & Tsien, 1993). Synthetic funnel-web spider toxin (sFTX 3.3) and serotonin (5-HT) were prepared as 10 mM stock solutions in distilled water. Purified ω-CTX GVIA, ω-CTX MVIIC and ω-Aga IVA were purchased from Alomone Labs (Jerusalem, Israel). Nifedipine was obtained from Interchim (Montluçon, France). sFTX 3.3 was a kind gift from Eli Lilly (Windelsham, UK) and was therefore similar to that used in the study of Scott *et al.* (1992). Serotonin, Mg-ATP, Na-GTP, guanosine 5′-*O*-thiotriphosphate (GTP-γ-S), pertussis toxin and cytochrome *c* were purchased from Sigma.

Expression of results

All results given in this paper are expressed as means ± S.D. (standard deviation).

The percentage (x) of inhibition of I_{Ca} by pharmacological agents was determined as:

$$x = (\Delta I/I_0) \times 100,$$

where ΔI is the amplitude of the current suppressed by a given blocking agent and I_0 is the the amplitude of the control current in the absence of any blocker.

In the experiments designed to identify the nature of the calcium current(s) inhibited by 5-HT, we determined the fraction of 5-HT effect (y) which was sensitive to the action of one or several specific Ca^{2+} channel blockers, by using the formula:

$$y = [(\Delta I_0 - \Delta I)/\Delta I_0] \times 100,$$

where ΔI_0 is the amplitude of the Ca^{2+} current suppressed by 5-HT under control conditions, i.e. in the absence of any Ca^{2+} channel blocker, and ΔI is the amplitude of the Ca^{2+} current suppressed by 5-HT in the presence of one (or several) Ca^{2+} channel blocking agents.

Dose–response curves of Ca^{2+} current inhibition by pharmacological agents

Dose–response curves were constructed by plotting the amplitude of the Ca^{2+} current (normalized with respect to the control current, i.e. in the absence of blocking agent) as a function of the concentration of the pharmacological agent (nifedipine or ω-Aga IVA). These substances were applied by bath perfusion and the steady-state holding potential was set at −70 mV. The amplitudes of the Ca^{2+} currents were corrected for run-down according to the method described by Randall & Tsien (1995). The experimental points were adjusted with either one-site (nifedipine) or two-site (ω-Aga IVA) binding curves using the program Graphpad Inplot (Graphpad software, San Diego, CA, USA). The equations used were those implemented in the software, i.e. for a single-site binding curve:

$$Y = A + (B - A)/(10^{x - \log C}),$$

and for a two-site binding curve:

$$Y = A + (B - A)[(F_1/10^{x - \log C_1}) + (F_2/10^{x - \log C_2})],$$

where Y is the amplitude of the Ca^{2+} current normalized with respect to the control current; A is the top limit of the curve; B is the bottom limit of the curve; x is the log of nifedipine or ω-Aga IVA concentration; C, C_1 and C_2 are the IC_{50} value(s) of the pharmacological agent for its binding site(s); F_1 is the fraction of binding sites having an IC_{50} of C_1; and F_2 is the fraction of binding sites having an IC_{50} of C_2 ($F_2 = 1 - F_1$).

RESULTS

Pharmacological properties of whole-cell Ca^{2+} currents

Melanotrophs are excitable endocrine cells which are known to possess at least two types of Ca^{2+} currents distinguishable on the basis of their kinetic properties and threshold of activation (Williams *et al.* 1990*a*; Keja & Kits, 1994*a*). In this study, a high-threshold or high voltage-activated (HVA) component of Ca^{2+} current, activated at membrane potentials more positive than −30 mV, was detected in all cells tested ($n = 42$). In addition, 92% of the cells examined also displayed a low-threshold or low voltage-activated (LVA) Ca^{2+} current having properties similar to that of the T-type current initially described in neurones (see review by Scott *et al.* 1991). Both types of current were carried by Ca^{2+} ions because they were reversibly abolished when Ca^{2+} was replaced by an equimolar amount of Co^{2+} in the extracellular solution.

In order to determine if the HVA Ca^{2+} current of rat melanotrophs consists of a single or of several distinct components, we have tested the effect of blockers of N-, L-, P- and Q-type channels. It is important to emphasize that none of the substances tested had any effect on the LVA Ca^{2+} current.

Effect of an N-type channel antagonist

The effect of ω-conotoxin GVIA (ω-CTX GVIA), which blocks N-type Ca^{2+} channels (McCleskey *et al.* 1987; Plummer *et al.* 1989; Kasai & Neher, 1992), was tested in cells possessing both LVA and HVA components of I_{Ca}. ω-CTX GVIA applied at concentrations of 0·5 μM ($n = 6$) or 1 μM ($n = 5$) for up to 15 min failed to inhibit the whole-cell calcium current recorded during a 100 ms voltage step from a holding potential (V_h) of −100 mV to a test

potential of 0 mV. These results indicated that rat melanotrophs do not possess N-type Ca^{2+} channels.

Effect of the L-type channel blocker nifedipine

Nifedipine (10 μM) inhibited 38·4 ± 10% ($n = 19$) of the whole-cell Ca^{2+} current during a 100 ms voltage step from a V_h of −70 mV to a test potential of 0 mV (Fig. 1*A*) but had no significant effect when V_h was set at −100 mV ($n = 4$). Nifedipine produced a parallel inhibition, i.e. a scaling down, of the total current in all cells tested ($n = 19$) suggesting that it suppressed a non-inactivating component of the Ca^{2+} current and that all melanotrophs possess functional L-type Ca^{2+} channels.

Figure 1*B* illustrates the complete dose–response relationship of nifedipine on the total Ca^{2+} current. In these experiments V_h was set at −70 mV. This relationship could be adjusted with a single binding site inhibition curve (see Methods section) with a half-maximal effective concentration (IC_{50}) of 28 nM. At nifedipine concentrations of 1 and 10 μM, the percentage block of the total I_{Ca} was comparable, indicating that at a concentration of 10 μM, nifedipine did not inhibit an additional Ca^{2+} current component in a non-specific manner. Our results are consistent with the blockade of a single class of L-type Ca^{2+} channels carrying about 39% of the total HVA calcium current.

Effect of ω-agatoxin IVA

At a concentration of 100 nM, ω-Aga IVA produced an irreversible and parallel reduction of I_{Ca} in a subset of cells (7 out of 12, i.e. 60%). The mean percentage inhibition of

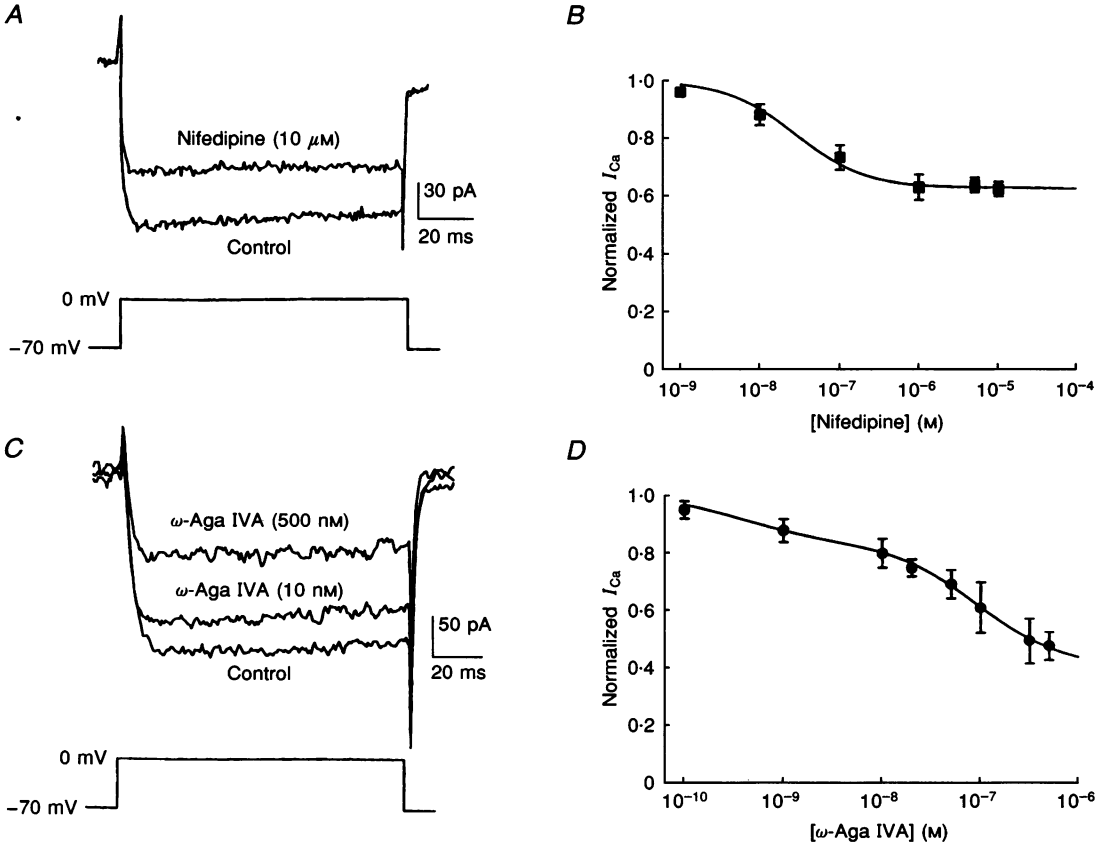

Figure 1. Effects of nifedipine and ω-Aga IVA on calcium currents of rat melanotrophs

Ca^{2+} currents were evoked by voltage steps to 0 mV from a holding potential (V_h) of −70 mV. *A*, inhibition of Ca^{2+} currents by nifedipine (10 μM). Note that nifedipine induced a parallel reduction of the current consistent with the suppression of a non-inactivating component of I_{Ca}. *B*, complete dose–response relationship for nifedipine. The amplitude of Ca^{2+} currents was normalized with respect to the control current (in the absence of nifedipine). The filled squares represent the means ± S.E.M. of 6–19 different cells. The curve has been fitted to the experimental points assuming a single binding site for nifedipine (see Methods). At maximally effective concentrations, nifedipine inhibited 39% of the total current. The IC_{50} value was 28 nM. *C*, effect of ω-Aga IVA on I_{Ca} at concentrations which block only P-type channels (10 nM) and both P- and Q-type channels (500 nM). *D*, complete dose–response relationship for ω-Aga IVA. The filled circles represent means ± S.E.M. from 5 cells. The curve is a best fit to the data points assuming two distinct binding sites (see Methods section). The maximal inhibition was of 61% and the values of the IC_{50} were of 0·4 and 91 nM, respectively.

I_{Ca} at a test potential of 0 mV was of 20 ± 10% ($n = 7$) and 37·6 ± 21·7% ($n = 5$) when V_h was set at −100 mV and −70 mV, respectively (Fig. 1*C*). This phenomenon can be explained by a larger contribution of ω-Aga IVA-sensitive components to the total I_{Ca} at a V_h of −70 mV compared with −100 mV.

The complete dose–response relationship of ω-Aga IVA on I_{Ca} is illustrated in Fig. 1*D*. This relationship was biphasic and the continuous line represents a fit of the experimental points with a two-component inhibition curve (see Methods for the equation) revealing a high affinity site ($IC_{50} = 0{\cdot}4$ nM) and a low affinity site ($IC_{50} = 91$ nM) for ω-Aga IVA. The high and low affinity components represented 26 and 74% of the ω-Aga IVA-sensitive current, respectively. The effect of ω-Aga IVA appeared to be maximal at concentrations above 300 nM. At saturating concentrations (>300 nM), ω-Aga IVA blocked about 61% of the total I_{Ca}, the remaining fraction of the HVA current being completely suppressed by 10 μM nifedipine ($n = 5$).

These high and low affinity components of the ω-Aga IVA-sensitive current could correspond to P-type and Q-type Ca^{2+} currents, respectively (Randall & Tsien, 1995; and see below).

Effect of sFTX 3.3

The effect of synthetic funnel-web spider toxin also known as synthetic arginine polyamine (sFTX 3.3; Scott *et al.* 1992) was tested at concentrations of 1 and 10 μM. Both concentrations gave similar percentages of inhibition of I_{Ca} suggesting that the effect of sFTX 3.3 was already maximal at 1 μM. Reduction of I_{Ca} by sFTX 3.3 was observed in a subset of cells (12 out of 16, i.e. 75%). The mean percentage inhibitions of I_{Ca} recorded at a potential of 0 mV were 25 ± 8% ($n = 8$) and 19 ± 7% ($n = 5$) when the holding potentials were set at −100 and −70 mV, respectively. The effect of sFTX 3.3 was dependent on the concentration of Ca^{2+} in the extracellular medium, i.e. increasing the extracellular Ca^{2+} concentration from 2 to 5 mM decreased the percentage inhibition of I_{Ca} from 25 ± 8% ($n = 7$) to 11 ± 1% ($n = 5$).

ω-Aga IVA occluded the effect of sFTX 3.3. We tested directly the additivity of ω-Aga IVA and sFTX 3.3 in five cells. In three cells ω-Aga IVA (100 nM) reduced I_{Ca} (by 16 ± 5%) and subsequent application of sFTX 3.3 (10 μM) had no additional effect. In the two remaining cells, neither ω-Aga IVA nor sFTX 3.3 had any effect on the whole-cell Ca^{2+} current. Moreover, when the cells were pre-incubated for 30 min to 1 h with ω-Aga IVA (50 nM), sFTX 3.3 (10 μM) had no effect on I_{Ca} in four out of four cells tested.

Pharmacological identification of a Q-type Ca^{2+} current in rat melanotrophs

Application of nifedipine (10 μM) together with sFTX 3.3 (1 μM) induced a 45 ± 9% ($n = 6$) reduction of the whole-cell calcium current, indicating that their effects were additive and that both agents blocked separate populations of channels. We noticed systematically the presence of a residual Ca^{2+} current component which was resistant to the combined effects of L- and P-type channel blockers. In order to identify this current, we have investigated in more detail its pharmacological properties.

The P-type current was suppressed by pre-incubating the cells for at least 1 h with 50 nM ω-Aga IVA. This concentration of ω-Aga IVA was chosen in order to ascertain complete block of P-type channels, although being aware that at this concentration the Q-type current was also partially blocked (see dose–response curve for ω-Aga IVA in the preceding section). During the experiment, nifedipine (10 μM) was added to the external medium in order to block L-type channels and ω-Aga IVA (50 nM) was present in the extracellular solution throughout the recording session. The steady-state holding potential was set at −70 mV in order to maintain the blocking action of nifedipine. Under these experimental conditions, a calcium current was detected in all cells tested ($n = 27$) and its mean amplitude, measured during a 100 ms voltage step to 0 mV, was 35 ± 30 pA ($n = 27$, range 10–138 pA). This current was carried by Ca^{2+} ions since it was completely and reversibly abolished when extracellular Ca^{2+} was replaced by an equimolar amount of Co^{2+} ($n = 5$).

Sensitivity to Cd^{2+} and Ni^{2+} ions

Figure 2*A* and *B* illustrates the effect of Cd^{2+} (10 μM) and Ni^{2+} (40 μM) on the Ca^{2+} current resistant to the L- and P-type channel blockers. In all cells tested ($n = 5$), we observed a complete block of I_{Ca} by Cd^{2+} (Fig. 2*A*) whereas in the same cells Ni^{2+} had no effect on the current (Fig. 2*B*). It must however be emphasized that in the absence of L- and P-type channel blockers, Ni^{2+} (40 μM) blocked 39 ± 5% of the total Ca^{2+} current in three out of three cells tested.

Effect of ω-conotoxin MVIIC

It has been recently reported that the marine snail toxin ω-CTX MVIIC blocks a fraction of Ca^{2+} current resistant to L-, P- and N-type channel blockers (Hillyard *et al.* 1992) and therefore identifies a novel type of Ca^{2+} current termed Q-type current (Zhang *et al.* 1993; Randall & Tsien, 1995). Figure 2*C* and *D* illustrates the effect of ω-CTX MVIIC and its time course on the Ca^{2+} current recorded in the presence of L- and P-type channel blockers. At a concentration of 1 μM, ω-CTX MVIIC blocked the residual Ca^{2+} current in an irreversible manner. The effect of ω-CTX MVIIC (1 μM) was usually maximal 6–10 min after onset of application. In five out of nine cells tested we observed a complete blockade of I_{Ca} by ω-CTX MVIIC (1 μM). In the remaining four cells, a small fraction of the peak I_{Ca} remained unblocked. This fraction corresponded probably to a part of the LVA current which had not been inactivated at a steady V_h of −70 mV, as suggested by the presence of a transient inward current during a voltage step to −30 mV.

Taken together our results indicate that the HVA calcium current of rat melanotrophs has multiple components. All cells possess functional L- and Q-type currents whereas only a subpopulation (~60–70%) also express P-type channels. In contrast N-type Ca^{2+} channels were not detected. Analysis of the dose–response curves for nifedipine and ω-Aga IVA suggests that L-, P- and Q-type components constitute 39, 16 and 45% of the total HVA calcium current, respectively.

Effect of 5-HT on the total Ca^{2+} current

5-HT (10 μM) reduced the whole-cell Ca^{2+} current in 84 out of 120 cells tested (70%) with little variability among the responsive cells in different cultures ($n = 28$). The effect of 5-HT was rapid in onset (<10 s), completely reversible and could be reproduced several times in the same cell without significant attenuation.

The modalities of calcium current (I_{Ca}) inhibition varied among cells and could be divided into three groups (Fig. 3). Figure 3*A* illustrates the case of a cell in which we observed a marked reduction of the peak inward calcium current associated with a slowing of the activation phase of the current. The degree of inhibition of I_{Ca} produced by 5-HT became progressively less important with time during the voltage step. Thus, the amplitudes of I_{Ca} in the absence and in the presence of 5-HT were almost identical just before termination of the 100 ms lasting voltage step to a test potential of 0 mV. This effect of 5-HT, which will be referred to as the kinetic slowing effect, was observed in

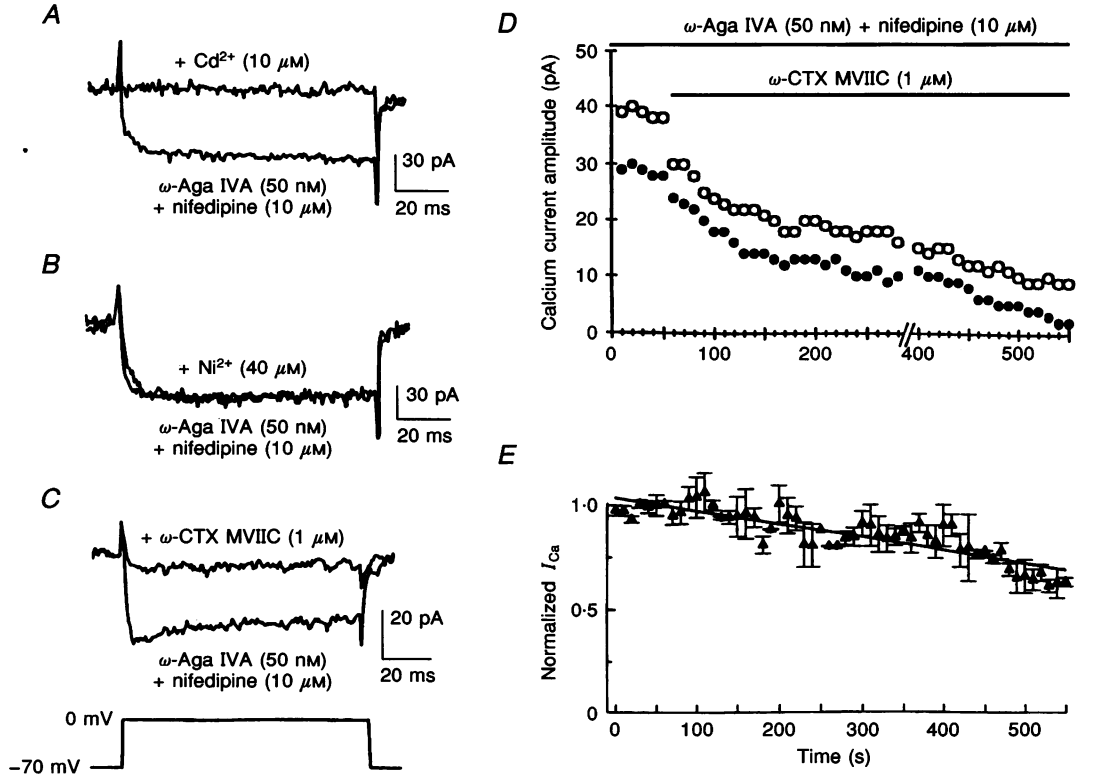

Figure 2. Pharmacological properties of the Q-type Ca^{2+} current

Calcium currents were evoked during 100 ms voltage steps from −70 to 0 mV every 10 s. All recordings were obtained after pre-incubation (>1 h) with ω-Aga IVA (50 nM) in order to block all P-type channels. Recordings were performed in the continuous presence of ω-Aga IVA (50 nM) and of nifedipine (10 μM), which blocks L-type Ca^{2+} channels. Note that the remaining current, which was essentially non-inactivating, was completely blocked by Cd^{2+} (10 μM) (*A*) but unaffected by Ni^{2+} (40 μM) (*B*). *C*, this current was largely reduced by the Q-type channel blocker ω-CTX MVIIC (1 μM) except a small fraction of transient current which probably corresponded to the LVA T-type current. *D*, time course of the effect of ω-CTX MVIIC (1 μM) in the same cell as in *C*. Open and closed circles represent the amplitudes of I_{Ca} measured at the peak and just before termination of the voltage step (sustained component), respectively. After 8 min (time 550 s) the sustained component of the current was completely blocked. *E*, run-down profile of the Q-type current. The amplitude of the calcium current (normalized with respect to the amplitude of the current at the beginning of the recording) is represented as a function of time. Each filled triangle represents the mean ± S.D. from 5 different cells. The continuous line is a linear regression indicating a rate of run-down of 3·5% min^{-1}. Note that the run-down was much slower than the blocking effect of ω-CTX MVIIC.

37% (31 out of 84) of the cells responding to 5-HT and the mean percentage inhibition of I_{Ca} was 27 ± 6% ($n = 31$). In contrast, 5-HT produced a scaling down, i.e. a parallel reduction, of I_{Ca} in 14% (12 out of 84) of the responsive cells (Fig. 3*B*). In this case, the amplitude of I_{Ca} suppressed at the begining and at the end of the depolarizing voltage step to 0 mV was the same and the mean percentage inhibition of I_{Ca} was 23 ± 7% ($n = 12$). Finally, in the remaining 49% (41 out of 84) of the cells, both types of effects were found to coexist (Fig. 3*C*). In these cells ($n = 41$) the reduction of I_{Ca} was of 34 ± 8% at the peak and of 17 ± 9% just before termination of the voltage step. As a consequence, the inhibition of I_{Ca} produced by 5-HT at the peak of the current represented the sum of both the kinetic slowing and scaling down effects, whereas the inhibition observed just before termination of the voltage step can be considered as a good index of the scaling down effect.

Identification of the 5-HT receptor subtypes involved in the inhibition of the Ca^{2+} current

In a previous study on cultured porcine melanotrophs (Ciranna *et al.* 1993), we have shown that the inhibitory effect of 5-HT was mediated by a dual population of receptors: 5-HT_{1A} and 5-HT_{1C}. Both receptors belong to the G-protein-coupled receptor family (Zifa & Fillion, 1992) and the 5-HT_{1C} receptor, because of its large sequence homology with the 5-HT_2 receptors, has been recently renamed 5-HT_{2C} (Hoyer *et al.* 1994). In order to determine if 5-HT_{1A} and 5-HT_{2C} receptors are also responsible for the inhibition of I_{Ca} in rat melanotrophs we tested the effect of several agonists and antagonists of these receptor subtypes.

Involvement of 5-HT_{2C} receptors

Mianserin (1 μM), an antagonist of 5-HT_2 receptors, was tested in six cells. In one cell, mianserin blocked completely the response to 5-HT (10 μM) whereas in the remaining cells it antagonized the 5-HT effect by 44 ± 21% (range, 20–73%). The effects of mianserin were rapidly and fully reversible. In contrast, ketanserin, at a concentration of 100 nM, at which it does not block 5-HT_{2C} receptors, had no effect on the 5-HT response ($n = 4$). These results indicate that at least part of the effect of 5-HT on I_{Ca} is mediated by a 5-HT_{2C} receptor subtype.

Involvement of 5-HT_{1A} receptors

The 5-HT_{1A} receptor agonist (±)8-hydroxy-(2-*N*,*N*-dipropylamino)-tetralin (8-OH-DPAT) at a maximally effective concentration of 100 nM (Ciranna *et al.* 1993) produced a reversible reduction of I_{Ca}. The mean percentage inhibition of the peak I_{Ca} was 25 ± 6% ($n = 14$). 8-OH-DPAT induced kinetic slowing (in 10 out of 14 cells) as well as scaling down (in 4 out of 14 cells) of I_{Ca}, suggesting that the modality of inhibition was not related to the activation of a given type of receptor.

These results suggest that rat melanotrophs possess a dual population of 5-HT_{1A} and 5-HT_{2C} receptors, the activation of which induces a reversible inhibition of the whole-cell Ca^{2+} current.

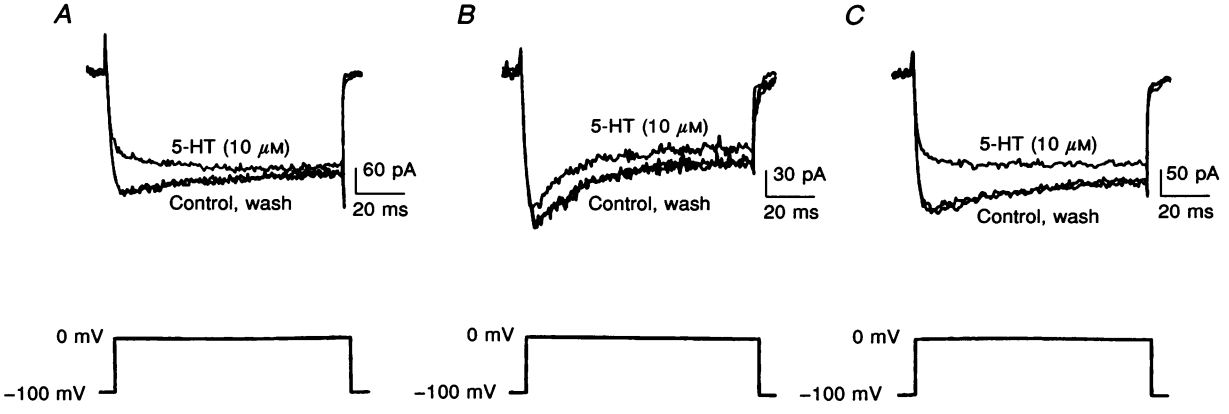

Figure 3. Effects of serotonin (5-HT) on Ca^{2+} currents in rat IL cells

Whole-cell Ca^{2+} currents were triggered by 100 ms voltage steps to 0 mV from a V_h of −100 mV, delivered every 10 s. 5-HT (10 μM, 30 s local application) reduced the amplitude of Ca^{2+} current, and two distinct patterns of inhibition were observed in different cells. *A*, 5-HT produced a kinetic slowing of the activation phase of I_{Ca} with no marked inhibition of the current just before termination of the voltage pulse (sustained I_{Ca}). *B*, in another cell, 5-HT caused a scaling down, i.e. parallel reduction, of I_{Ca} suggesting that only a non-inactivating component was affected. *C*, coexistence of kinetic slowing and scaling down effects of 5-HT on I_{Ca} in the same cell. 5-HT produced both a slowing in activation of peak I_{Ca} and inhibition of the sustained component. Effects of 5-HT were fully reversible. Traces labelled Control and wash correspond to Ca^{2+} current traces recorded before application and after wash-out of 5-HT.

Effect of 5-HT on the Ca^{2+} current $I-V$ relationship

Figure 4. shows a typical effect of 5-HT (10 μM) on the current–voltage ($I-V$) relationship of the whole-cell Ca^{2+} current. In this cell, 5-HT induced a kinetic slowing as well as a reduction in amplitude of I_{Ca} just before termination of the 100 ms voltage step from −100 to −10 mV (Fig. 4*A*). The reduction of I_{Ca} at the peak could not be explained by an inhibition of the transient LVA Ca^{2+} current since 5-HT had no effect on this component recorded in isolation during a voltage step to −30 mV (Fig. 4*B*). The complete $I-V$ relationships of the whole-cell Ca^{2+} current in the absence (open circles) and in the presence of 5-HT (filled circles) are shown in Fig. 4*C*. 5-HT (10 μM) selectively inhibited the high threshold component of I_{Ca}, i.e. at membrane potentials >−30 mV. The maximal effect of 5-HT was usually observed at potentials of −10 or 0 mV. At more depolarized potentials the degree of inhibition became progressively smaller and no inhibition was observed at potentials more positive than +20 mV. Similar observations were made in five cells.

These results indicated that 5-HT selectively inhibited one or several components of the HVA Ca^{2+} current and that this effect was strongly dependent on membrane potential. This observation was true for both scaling down and kinetic slowing effects of 5-HT.

Voltage dependence of the 5-HT effect

To test the voltage dependence of the 5-HT effect, we used a prepulse protocol consisting of a 30 ms conditioning voltage step which preceded a 100 ms test voltage step to 0 mV. The steady-state holding potential in these experiments was −100 mV. Both voltage steps (conditioning and test) were separated by a 10 ms period during which the holding potential was returned to −100 mV. Figure 5*A* shows a typical result. In the absence of a conditioning step, 5-HT (10 μM) reversibly reduced I_{Ca} involving both kinetic slowing and scaling down effects, the latter being attested by a persistent inhibition of I_{Ca} just before termination of the voltage step (Fig. 5*A*, left panel). The effect of 5-HT was strongly reduced by a conditioning prepulse to +80 mV (Fig. 5*A*, middle panel) in a reversible

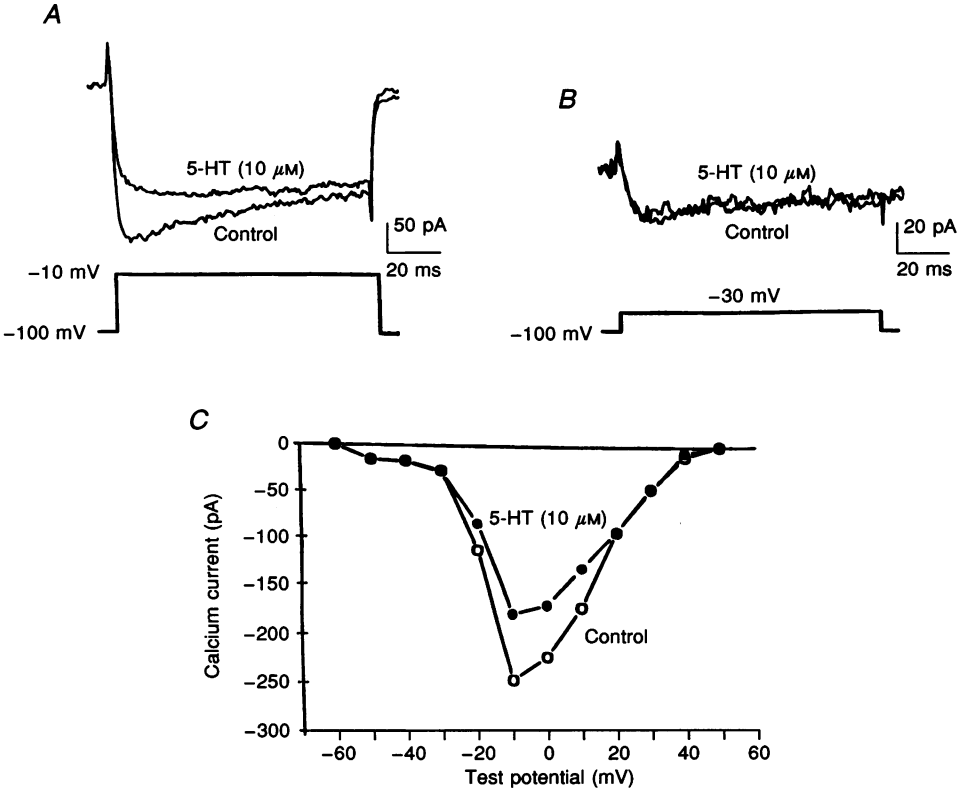

Figure 4. Effect of 5-HT on the current–voltage ($I-V$) relationship of Ca^{2+} currents

A, effect of 5-HT (10 μM, 30 s application by microperfusion) on whole-cell Ca^{2+} current triggered by 100 ms voltage steps to −10 mV from a V_h of −100 mV. Note the presence of both kinetic slowing and scaling down components of the 5-HT effect. *B*, 5-HT did not affect the LVA Ca^{2+} current, evoked by voltage steps to −30 mV from a V_h of −100 mV. *C*, current–voltage relationship for total peak Ca^{2+} current recorded in control conditions (○) and during application of 5-HT (●). Two components corresponding to the activation of LVA (>−60 mV) and HVA (>−30 mV) Ca^{2+} currents are observed. 5-HT (10 μM) selectively inhibited the HVA Ca^{2+} current but did not affect the LVA component. All records are from the same cell.

manner (Fig. 5*A*, right panel). In eleven cells tested with such a protocol, the 30 ms conditioning step to +80 mV reduced the effect of 5-HT by $80 \pm 20\%$ ($n = 11$). Figure 5*B* illustrates in more detail the consequences of varying the voltage of the prepulse on the percentage inhibition of I_{Ca} by 5-HT (10 μM) in four cells in which we observed a total abolition of the 5-HT effect following a pre-depolarization to +80 mV. The conditioning voltage step began to significantly reduce the effect of 5-HT at potentials more depolarized than −30 mV and the response to 5-HT was completely abolished beyond + 20 mV. As illustrated in Fig. 5*C*, the scaling down effect was also completely abolished by a pre-depolarization to +80 mV.

These results indicated that both the scaling down and the kinetic slowing effects of 5-HT on I_{Ca} were voltage dependent.

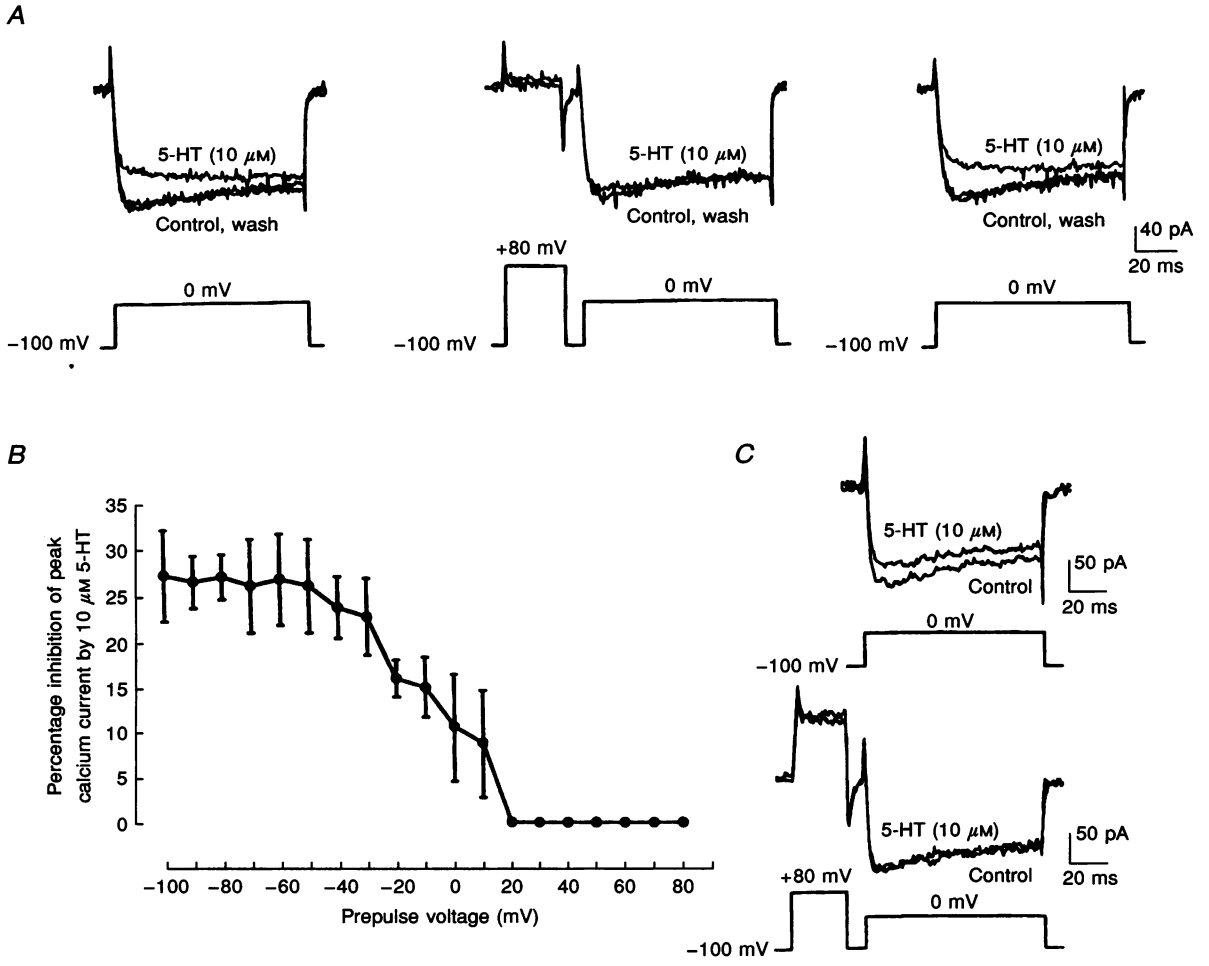

Figure 5. Voltage dependence of serotonin effect on Ca^{2+} current

A, left panel, reversible inhibition of Ca^{2+} current by 5-HT (10 μM, 30 s application). Ca^{2+} currents were elicited by 100 ms voltage steps to 0 mV from a V_h of −100 mV. Middle panel, 5-HT-mediated inhibition of Ca^{2+} current was completely suppressed by a 30 ms depolarizing prepulse to +80 mV preceding the test pulse to 0 mV. Right panel, 5-HT effect on Ca^{2+} currents was fully recovered when returning to the standard protocol, i.e. in the absence of any prepulse. Traces labelled Control and wash represent Ca^{2+} currents recorded before application and after wash-out of 5-HT, respectively. *B*, a double-pulse protocol was used to investigate the effects of increasing depolarizing prepulses on 5-HT-mediated inhibition of Ca^{2+} currents. Test pulses (from −100 to 0 mV, during 100 ms) were preceded by prepulses ranging from −100 to +80 mV (30 ms in duration). The amplitude of Ca^{2+} currents evoked by test pulses was measured before and during application of 5-HT, and the percentage inhibition of peak Ca^{2+} current was plotted against prepulse voltage. Each point is the mean from 4 different cells and the error bars represent the standard deviations. A prepulse to −20 mV reduced by 40% the effect of 5-HT on peak Ca^{2+} current evoked by the test pulse. The effect of 5-HT was reduced by 67% by a prepulse to +10 mV, and completely abolished by a prepulse to +20 mV ($n = 4$). *C*, example of a cell in which 5-HT had an exclusively scaling down effect on I_{Ca}. Note that this effect of 5-HT was completely abolished by a depolarizing prepulse to +80 mV.

Involvement of a G-protein

Effect of GTP-γ-S

When the cells were dialysed with an electrode solution containing the non-hydrolysable GTP analogue GTP-γ-S (30 μM), 5-HT (10 μM) inhibited I_{Ca} by 33 ± 12% in five out of twelve cells tested. In all cases, the effect of 5-HT was irreversible and a second application of 5-HT was ineffective in producing further reduction of I_{Ca}.

Effect of pertussis toxin (PTX)

After pretreatment of the cells with PTX (150 ng ml^{-1}) for 18 h, we never observed any effect of 5-HT in eight out of eight cells tested whereas 5-HT still reduced I_{Ca} in five out of eight cells in untreated sister cultures. It is important to mention that the amplitudes of calcium currents in PTX-treated and control (untreated) cells were similar: 136 ± 49 pA ($n = 8$) and 114 ± 32 pA ($n = 8$), respectively.

These results indicated that 5-HT inhibited I_{Ca} by a mechanism involving a G-protein of the G_i or G_o type.

Identification of the Ca^{2+} current subtypes inhibited by 5-HT

In order to determine which components of the HVA Ca^{2+} current were modulated by 5-HT, we tested the effect of L-, P, and Q-type channel blockers on the inhibition of I_{Ca} mediated by 5-HT.

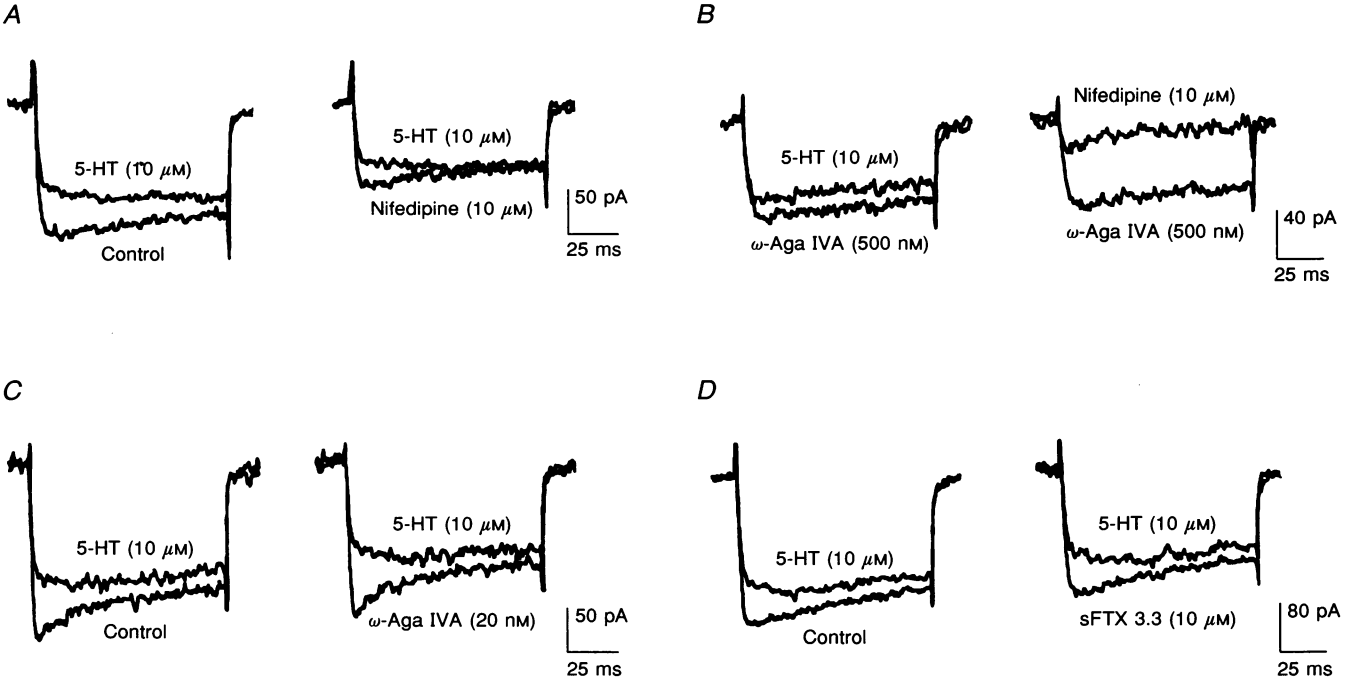

Figure 6. 5-HT inhibits L-type but not P-type Ca^{2+} currents

The Ca^{2+} currents were activated by 100 ms voltage steps to 0 mV from a V_h of −70 mV (*A*, *B* and *C*) or −100 mV (*D*). *A*, effect of the L-type channel blocker nifedipine (10 μM). Under control conditions (left traces), 5-HT (10 μM) inhibited I_{Ca} both at the peak (kinetic slowing effect) and just before termination of the voltage step (scaling down effect). Nifedipine reduced the total current by about 40% and completely suppressed the inhibition of I_{Ca} by 5-HT observed at the end of the voltage step (right traces). *B*, application of ω-Aga IVA at a concentration of 500 nM (left traces) in order to suppress both P-type and Q-type currents blocked about 60% of the total I_{Ca}. The current remaining under these conditions (right traces) was scaled down by 5-HT (10 μM) and blocked by nifedipine (10 μM), indicating that it was an L-type current. The fraction of current remaining unblocked has a transient time course and corresponds to the T-type LVA Ca^{2+} current. *C*, effect of a low concentration of ω-Aga IVA (20 nM) on the 5-HT effect. At the concentration used, ω-Aga IVA blocks relatively specifically P-type channels. Note that ω-Aga IVA inhibited the total I_{Ca} by about 20% but did not affect the fraction of calcium current suppressed by 5-HT (left traces: control; right traces: in the presence of 20 nM ω-Aga IVA). This observation suggested that 5-HT did not modulate P-type currents. *D*, effect of sFTX 3.3, which also blocks P-type channels. The amplitudes of the current suppressed by 5-HT (10 μM) under control conditions (left traces) and in the presence of 10 μM sFTX 3.3 (right traces) were the same, although sFTX 3.3 reduced the total I_{Ca} by about 20%. Note that these results were similar to those obtained with 20 nM ω-Aga IVA.

Effect of nifedipine

The effect of nifedipine (10 μM) was tested in seven cells responding to 5-HT (10 μM). In three cells which displayed exclusively a kinetic slowing in response to 5-HT, nifedipine had no effect on the amplitude of the current component suppressed by 5-HT, although in the same cells nifedipine reduced the total I_{Ca} by $31 \pm 4\%$ ($n = 3$). Indeed, in these cells the amplitudes of the currents suppressed by 5-HT were 34, 24 and 17 pA under control conditions and 35, 23 and 18 pA in the presence of 10 μM nifedipine. In three other cells in which both kinetic slowing and scaling down effects coexisted, nifedipine blocked $56 \pm 7\%$ ($n = 3$) of the effect of 5-HT at the peak (where both modalities of inhibition contributed to the reduction of I_{Ca}) and abolished the inhibition of the sustained component of I_{Ca} observed just before termination of the voltage step (which reflects mainly the scaling down effect of the 5-HT, Fig. 6*A*). Consistent with this, nifedipine completely suppressed the effect of 5-HT in a cell in which 5-HT had induced an exclusively scaling down effect of I_{Ca} under control conditions (i.e. in the absence of nifedipine). In addition, in eight out of eight cells which responded to 5-HT in the presence of nifedipine (10 μM), we observed exclusively a kinetic slowing type of inhibition of I_{Ca} and no reduction of the Ca^{2+} current just before termination of the voltage step. Moreover, in the presence of 500 nM ω-Aga IVA, which blocks totally P- and Q-type currents, 5-HT induced an exclusively scaling down effect of the residual Ca^{2+} current in all cells tested ($n = 4$, Fig. 6*B*). This current was in turn blocked by nifedipine (10 μM) indicating that it was an L-type current.

These results suggested that the inhibition of an L-type Ca^{2+} current by 5-HT accounted for the scaling down effect but that the kinetic slowing effect was not due to the modulation of L-type Ca^{2+} channels.

Effect of P-type Ca^{2+} channel blockers

Pre-incubation of the cultures with ω-Aga IVA (50 nM) did not affect the fraction of cells responding to 5-HT and the percentage inhibition of I_{Ca} by 5-HT was $36 \pm 3\%$ ($n = 6$). In the presence of a low concentration of ω-Aga IVA (20 nM), which blocks relatively selectively all P-type channels ($IC_{50} = 0{\cdot}4$ nM, see Fig. 1*D*) and only a small fraction of Q-type channels (approximately 10% as estimated from the analysis of the dose–response curve shown in Fig. 1*D*), we observed no reduction of the amplitude of the Ca^{2+} current suppressed by 5-HT compared with that suppressed in the same cell under control conditions, i.e. in the absence of ω-Aga IVA ($n = 7$, Fig. 6*C*). In seven cells tested, the mean amplitudes of the current suppressed by 5-HT at the peak and just before termination of the voltage step were of 38 ± 8 and $10{\cdot}2 \pm 2$ pA, respectively, under control conditions and of $36{\cdot}8 \pm 8$ and $10{\cdot}8 \pm 2$ pA, respectively, in the presence of 20 nM ω-Aga IVA. In the same cells, ω-Aga IVA (20 nM) inhibited the total I_{Ca} by $26{\cdot}3 \pm 7{\cdot}9\%$ ($n = 7$).

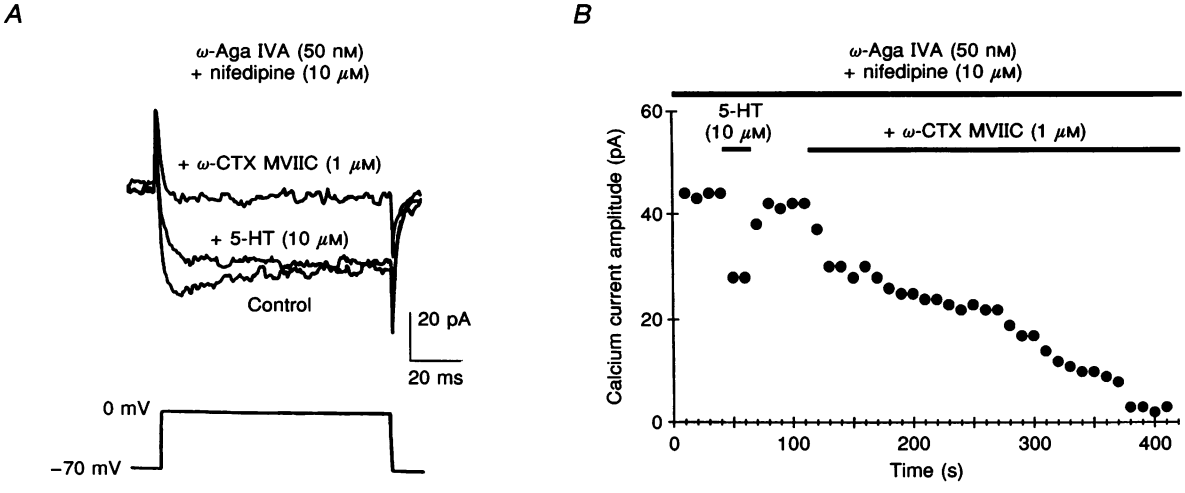

Figure 7. Selective inhibition of a Q-type Ca^{2+} current by 5-HT

A, the control current was recorded after pre-incubation of the cell with ω-Aga IVA (50 nM) for 1 h in order to block P-type channels. At the beginning of the recording, nifedipine (10 μM) was added to the external medium in order to block L-type channels. ω-Aga IVA (50 nM) was present throughout the experiment. The current, which was resistant to both L- and P-type channel blockers, was still inhibited by 5-HT (10 μM). Note that the effect of 5-HT was exclusively of the kinetic slowing type. Application of ω-CTX MVIIC (1 μM) completely abolished this Ca^{2+} current indicating that it was due to Q-type channels. *B*, time course of the effects of 5-HT (10 μM) and ω-CTX MVIIC (1 μM) on the peak Ca^{2+} current in the cell illustrated in *A*.

sFTX 3.3 at a maximally effective concentration (1–10 μM) had no significant effect on the 5-HT-induced inhibition of I_{Ca}, the mean reduction of the amplitude of the 5-HT response by sFTX 3.3 being 6 ± 9% ($n = 9$, Fig. 6*D*).

Effect of ω-conotoxin MVIIC

The results presented in the preceding sections suggested that the kinetic slowing effect induced by 5-HT was not due to the modulation of L- or P-type calcium channels. Thus, it was likely that this effect of 5-HT reflected the inhibition of the Q-type calcium current. This was directly confirmed by the type of experiment illustrated in Fig. 7. The cells were pre-incubated for 1–2 h with ω-Aga IVA (50 nM) and whole-cell calcium currents were recorded after addition of nifedipine (10 μM) to the extracellular medium, which still contained ω-Aga IVA (50 nM). Under these conditions, L- and P-type currents and probably about one-third of the Q-type currents were blocked and 5-HT produced an exclusively kinetic slowing effect of the residual current. The mean inhibition of I_{Ca} was 35 ± 6% ($n = 8$) and the effect of 5-HT was fully reversible. In all cells where it was tested ($n = 5$), application of ω-CTX MVIIC (1 μM) reduced the Ca^{2+} current by 85 ± 13% (range, 70–100%) and abolished completely the effect of 5-HT. Moreover in the presence of 500 nM ω-Aga IVA, which blocks completely P-type and Q-type currents (see Fig. 1*D*), we never observed kinetic slowing effects ($n = 4$, see Fig. 6*B*).

These results indicated that 5-HT was inhibiting a Q-type current by a kinetic slowing effect.

DISCUSSION

The two major objectives of this work were (i) to identify the subtypes of Ca^{2+} channels underlying the whole-cell high voltage-activated (HVA) Ca^{2+} current, and (ii) to study their modulation by 5-HT, in the endocrine cells of the rat intermediate pituitary (melanotrophs). The use of blockers of different Ca^{2+} channel subtypes allowed us to infer the presence of functional L- and P-type calcium channels, and most importantly of Q-type Ca^{2+} channels, which have, to our knowledge, never been identified in primary endocrine cells and have been detected only recently in neurones (Wheeler, Randall & Tsien, 1994; Randall & Tsien 1995). In addition, our results suggest that 5-HT selectively modulates L- and Q-type, but not T- and P-type, calcium channels with different modalities but by a mechanism involving a pertussis toxin-sensitive G-protein.

Composition of HVA calcium current in rat melanotrophs

L-type channels

We confirm the results of earlier studies showing the presence of a dihydropyridine-sensitive L-type current in melanotrophs (e.g. Williams *et al.* 1990*a*; Stack & Surprenant, 1991; Keja & Kits, 1994*a*). Analysis of the dose–response profile of the effect of nifedipine on the total I_{Ca} in our model suggests the existence of a single population of L-type channels ($IC_{50} = 28$ nM), which accounts for 39% of the whole-cell calcium current when the steady-state holding potential was set at −70 mV. L-type currents were present in all cells tested.

N-type channels

ω-CTX GVIA, which blocks specifically N-type Ca^{2+} channels (Plummer *et al.* 1989; Kasai & Neher, 1992), had no effect on the total Ca^{2+} current indicating the absence of N-type currents. These findings agree with the results of other electrophysiological studies on Ca^{2+} currents in rat melanotrophs (Wang, Treistman & Lemos, 1992; Williams, Pittman & MacVicar, 1993; but see Stack & Surprenant, 1991) including those at the single-channel level (Keja & Kits, 1994*a*).

P-type and Q-type channels

ω-Aga IVA blocks P-type channels at low nanomolar concentrations ($IC_{50} = 1$–2 nM; Mintz *et al.* 1992) whereas at higher concentration it also blocks Q-type currents (Randall & Tsien, 1995). Analysis of dose–response curves for the inhibition of I_{Ca} by ω-Aga IVA revealed the presence of a high affinity site ($IC_{50} = 0{\cdot}4$ nM) and a low affinity site ($IC_{50} = 91$ nM) for this toxin in rat melanotrophs. These results are similar to those reported recently by Randall & Tsien (1995) for P- and Q-type currents in cultured rat cerebellar granule neurones (IC_{50} of 1 and 89 nM, respectively). At a steady-state holding potential of −70 mV, the high affinity (P-type) and the low affinity (Q-type) components represented 26 and 74%, respectively, of the ω-Aga IVA-sensitive current in rat melanotrophs. Since, at saturating concentrations, ω-Aga IVA blocked 61% of the total I_{Ca}, we calculated that P- and Q-type current components represented respectively 16 and 45% of the whole-cell Ca^{2+} current. A component similar to the P-type current was inhibited by sFTX 3.3. This component represented 19% of the total I_{Ca} and the effect of sFTX was occluded by ω-Aga IVA. Most importantly the P-type current was detected only in a subset of melanotrophs (60–70%).

The most interesting finding of our study is the presence of Q-type Ca^{2+} channels in all cells tested. In order to study the Q-type current in isolation, L- and P-type currents were completely blocked with 10 μM nifedipine and 50 nM ω-Aga IVA, respectively. The use of a relatively high concentration of ω-Aga IVA was necessary in order to be sure that all P-type channels were blocked before testing the effect of ω-CTX MVIIC, which inhibits Q-type channels but also N- and P-type channels (Hillyard *et al.* 1992; Randall & Tsien, 1995). Of course at the concentration of ω-Aga IVA used in these experiments (50 nM) about one-third of the Q-type current was also blocked. Under these experimental conditions, the residual Ca^{2+} current was blocked by ω-CTX MVIIC (1 μM) and 10 μM Cd^{2+} but was insensitive to 40 μM Ni^{2+}. In addition, this current was never observed in the presence of high

concentrations of ω-Aga IVA (300–500 nM). These pharmacological properties suggested that this current was a Q-type Ca^{2+} current.

Effect of 5-HT on I_{Ca}

Serotonin inhibited the HVA Ca^{2+} current in about 70% of cultured rat melanotrophs, a percentage comparable to that of 5-HT-responsive cells in porcine intermediate lobe cells (Ciranna *et al.* 1993). As in porcine melanotrophs, the LVA Ca^{2+} current was unaffected by 5-HT and the inhibition of I_{Ca} was mediated by a dual population of receptors ($5\text{-}HT_{1A}$ and $5\text{-}HT_{2C}$) and a pertussis toxin-sensitive G-protein.

There was, however, a marked difference between rat and porcine melanotrophs concerning the modality of Ca^{2+} current inhibition by 5-HT. In porcine melanotrophs the effect of 5-HT was characterized exclusively by a parallel, i.e. scaling down-type, inhibition of the whole-cell Ca^{2+} current. In the rat, we observed a second type of inhibition consisting of a slowing of the activation phase of the Ca^{2+} current (kinetic slowing effect). This effect was similar to that described for the inhibition of HVA calcium currents in rat dorsal raphe neurones by 5-HT (Penington *et al.* 1991). Although each modality of Ca^{2+} current inhibition could be observed in isolation in a subset of melanotrophs, i.e. 37% of the cells for the kinetic slowing effect and 14% of the cells for the scaling down effect, they coexisted in the majority (49%) of the 5-HT-responsive cells. It is also important to note that a given modality of Ca^{2+} current inhibition was not linked to the activation of a given subtype of 5-HT receptor, since 8-OH-DPAT, a selective agonist of the $5\text{-}HT_{1A}$ receptor, induced both kinetic slowing and scaling down effects.

Involvement of a G-protein in the effect of 5-HT

Both kinetic slowing and scaling down effects of 5-HT on Ca^{2+} current inhibition were mediated via the activation of a G-protein for the following reasons. Firstly, both effects of 5-HT were completely abolished by pretreatment of the cells with pertussis toxin (PTX) which inactivates G-proteins of the G_i and G_o families (Schultz, Rosenthal, Hescheler & Trautwein, 1990; Scott *et al.* 1991). Secondly, the effects of 5-HT, which are normally rapidly reversible, were rendered irreversible by the inclusion of the non-hydrolysable GTP analogue GTP-γ-S, which tonically activates the G-protein (Scott *et al.* 1991). Finally, the effects of 5-HT were reversed by a depolarizing prepulse, a phenomenon which is also characteristic of G-protein-mediated modulation of voltage-dependent ion channels (Bean, 1989; Penington *et al.* 1991; Elmslie, Kammermeier & Jones, 1992; Ikeda, 1992; Golard & Siegelbaum, 1993). Moreover, this point argues in favour of a direct interaction between the G-protein and the ion channel (Lopez & Brown, 1991; Scott *et al.* 1991; Golard & Siegelbaum, 1992; Ikeda, 1992).

Kinetic slowing effect of 5-HT on I_{Ca}

In neurones, a major target for neurotransmitter-induced modulation of HVA Ca^{2+} currents is the ω-CTX GVIA-sensitive N-type Ca^{2+} current (Beech, Bernheim & Hille, 1992; Elmslie *et al.* 1992; Ikeda, 1992; Boland & Bean, 1993; Golard & Siegelbaum, 1993). In this type of inhibition, the neurotransmitter shifts the voltage dependence of N-type Ca^{2+} channels towards more depolarized potentials (Bean, 1989). This effect is responsible for the slowing of the rising phase of the Ca^{2+} current and can be overcome by strong membrane depolarization (e.g. Elmslie *et al.* 1992; Ikeda, 1992; Boland & Bean, 1993; Golard & Siegelbaum, 1993). Although kinetic slowing of the activation phase of the HVA calcium current was apparent in most of the 5-HT-responsive cells (86%), it could certainly not be attributed to the inhibition of an N-type Ca^{2+} current, since rat melanotrophs do not possess ω-CTX GVIA-sensitive Ca^{2+} channels (Wang *et al.* 1992; Williams *et al.* 1993; Keja & Kits, 1994*a*; and this report). Thus this effect must reflect the modulation of a Ca^{2+} current component which is different from an N-type current (see below), but by a mechanism which closely resembles that of inhibition of N-type channels by neurotransmitters in neurones.

Scaling down of I_{Ca} by 5-HT

Scaling down of Ca^{2+} currents by neurotransmitters has been described in neurones (Docherty & McFadzean, 1989; Sayer, Schwindt & Crill, 1992) but is relatively rare compared with the kinetic slowing type of inhibition. In contrast it is a commonly observed modality of Ca^{2+} current inhibition in endocrine cells (Schultz *et al.* 1990; Kleuss, Hescheler, Ewel, Rosenthal, Schultz & Wittig, 1991; Kramer, Kaczmarek & Levitan, 1991). This type of inhibition seems to target essentially the L-type Ca^{2+} current and can involve both a direct inhibition of the channels by a membrane-delimited pathway via the activation of a pertussis toxin-sensitive G-protein (Kleuss *et al.* 1991; Beech *et al.* 1992; Sayer *et al.* 1992; Sahara & Westbrook, 1993) and a PTX-insensitive pathway mediated by a diffusible intracellular messenger (Kramer *et al.* 1991; Beech *et al.* 1992). In our system, inhibition of L-type Ca^{2+} channels totally accounts for the scaling down effect of 5-HT on I_{Ca} (see below). Interestingly, this effect of 5-HT is, like the kinetic slowing effect, strongly voltage dependent and abolished by pretreatment with pertussis toxin.

Relationship between the type of inhibition and the different components of the high threshold Ca^{2+} current

The use of blockers of different subtypes of Ca^{2+} channels allowed us to assign a given type of inhibition to a particular type of Ca^{2+} current. The scaling down effect was specifically blocked by the L-type channel blocker nifedipine whereas the kinetic slowing type of inhibition

persisted after blockade of L- and P-type Ca^{2+} currents but was completely suppressed by ω-CTX MVIIC, which under these conditions inhibited the Q-type calcium current (Zhang *et al.* 1993, Randall & Tsien, 1995). To our knowledge, this is the first demonstration of a G-protein-mediated inhibition of the Q-type Ca^{2+} current in an endocrine cell. Interestingly, Wheeler *et al.* (1994) reported recently that the synaptic release of neurotransmitter induced by activation of Q-type Ca^{2+} channels could be modulated by several neurotransmitters acting via receptors which belong to the G-protein-coupled receptor family. In dorsal raphe neurones, 5-HT also inhibits the Ca^{2+} current recorded in the presence of L-type and N-type channel blockers (Penington *et al.* 1991). It is however, not clear if this component of I_{Ca} corresponds to P-type and/or Q-type currents.

Physiological significance

Our results show that rat melanotrophs possess HVA L-, P- and Q-type Ca^{2+} channels which coexist with LVA Ca^{2+} channels. The properties of these Ca^{2+} channels will therefore define the properties of the Ca^{2+}-dependent action potentials of these cells (Williams *et al.* 1990*a*) and determine the profile of Ca^{2+} influx, which is directly linked to Ca^{2+}-dependent hormonal secretion (Nemeth, Taraskevich & Douglas, 1990). The LVA current seems to play an important role in the initiation of the Ca^{2+} spikes (Williams *et al.* 1990*a*) and it could well be that the three high-threshold components of Ca^{2+} current, which display little inactivation with time, are of fundamental importance in supporting sustained Ca^{2+} influx during depolarization beyond −30 mV. As a consequence the modulation of such Ca^{2+} currents by neurotransmitters might be an essential step for controlling hormonal secretion. In line with this, we have shown that rat melanotrophs possess functional 5-HT receptors which reversibly and selectively inhibit L- and Q-type Ca^{2+} currents through a voltage-dependent mechanism involving a pertussis toxin-sensitive G-protein. In a physiological context, 5-HT might be released from nerve fibres innervating the intermediate lobe of the rat pituitary (Mezey *et al.* 1984) to activate these receptors and produce inhibition of specific components of the HVA Ca^{2+} current as it has been described for the synaptic release of dopamine during electrical stimulation of the pituitary stalk (Williams *et al.* 1990*b*).

However, a major difference between the effects of 5-HT and dopamine is that 5-HT does not inhibit the LVA Ca^{2+} current and might therefore modulate selectively Ca^{2+} influx through HVA Ca^{2+} channels without interfering with the generation of Ca^{2+} spikes. Moreover, by analogy with the effect of 5-HT, it seems important to re-examine the effect of dopamine on the HVA Ca^{2+} current knowing that melanotrophs possess L-, P- and Q-type but not N-type Ca^{2+} channels. Interestingly, a recent report by Keja & Kits (1994*b*) indicates that the characteristics of the voltage-dependent inhibition of a HVA Ca^{2+} current component by dopamine in rat melanotrophs is very similar to the kinetic slowing effect of 5-HT on Q-type currents that we describe here. Therefore it could be that dopamine and 5-HT modulate the same subtypes of HVA Ca^{2+} channels in melanotrophs. The fact that Q-type Ca^{2+} channels are specific targets for the modulatory action of neurotransmitters already indicates that the Q-type current may be of fundamental importance in mediating and controlling hormonal secretion in melanotrophs. It is tempting to speculate that in endocrine cells the Q-type current may have a function similar to that described in hippocampal neurones in which it seems to play a prominent role in the synaptic release of neurotransmitter (Wheeler *et al.* 1994).

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