Ca^{2+} current expression in pituitary melanotrophs of neonatal rats and its regulation by D_2 dopamine receptors

Juan Carlos Gomora, Guillermo Avila and Gabriel Cota*

Department of Physiology, Biophysics and Neuroscience, Center for Research and Advanced Studies, National Polytechnic Institute, Mexico City, DF 07000, Mexico

1. We have examined the voltage-dependent Ca^{2+} channel activity of rat melanotrophs during the early postnatal period. The cells were dissociated from pituitary intermediate lobes, kept in culture for 5–24 h and then subjected to whole-cell patch-clamp experiments.

2. Like their adult counterparts, neonatal melanotrophs were able to generate Na^+ currents, K^+ currents and Ca^{2+} currents in response to membrane depolarization. Ca^{2+} currents were carried by both low- and high-threshold Ca^{2+} channels.

3. High-threshold Ca^{2+} current density decreased sharply between postnatal day 4 (P4) and P12. This period coincides with the onset of dopaminergic innervation within the intermediate lobe. Accordingly, the developmental decrease in Ca^{2+} current density was largely reversed by chronic *in vivo* treatment with sulpiride, a dopamine D_2 receptor antagonist.

4. Prolonging the time in culture from 5 h to 8 days did not significantly alter the Ca^{2+} channel activity of P3 melanotrophs, whereas the high-threshold Ca^{2+} current in previously innervated (P14) melanotrophs stayed small for the first 24 h and then increased 3-fold during the subsequent 4–5 days. This increase required RNA and protein synthesis and was prevented by adding D_2 agonists to the culture medium.

5. These results provide evidence for a postnatal suppression of high-threshold Ca^{2+} current expression in pituitary melanotrophs mediated by presynaptic dopamine neurons through D_2 dopamine receptors.

It is well established that presynaptic neurons play important roles in inducing developmental changes in the expression and function of ion channels in postsynaptic muscle cells (e.g. Sherman & Catterall, 1982; Gonoi & Hasegawa, 1988; Gu & Hall, 1988; Gonoi, Hagihara, Kobayashi, Nakamura & Ohizumi, 1989; Zhang, Robinson & Siegelbaum, 1992). However, the regulatory influence of innervation on the development of ion channel activity in endocrine cells has not been addressed thus far. To begin to examine this topic, we have carried out an electrophysiological study on pituitary melanotrophs from neonatal and immature rats.

Melanotropes account for more than 95% of the cells found in the intermediate lobe (IL) of the rat pituitary (Chronwall, Millington, Griffin, Unnerstall & O'Donohue, 1987). They synthesize pro-opiomelanocortin (POMC) and secrete α-melanocyte-stimulating hormone (α-MSH), β-endorphin and other POMC-derived peptides. In the adult stage, the melanotrophs are primarily regulated by dopaminergic neurons that originate in the mediobasal hypothalamus and directly innervate the IL (Holzbauer & Racke, 1985; Goudreau, Lindley, Lookingland & Moore, 1992). Dopamine released from the nerve terminals tonically inhibits the synthesis and release of POMC peptides (Millington & Chronwall, 1989), acting through D_2 dopamine receptors (Munemura, Cote, Tsuruta, Eskay & Kebabian, 1980; Mansour, Meador-Woodruff, Bunzow, Civelli, Akil & Watson, 1990). Functional D_2 receptors appear early in the developing rat IL and are already present before birth (Davis, Lichtensteiger, Schlumpf & Bruinink, 1984). However, newborn rat melanotrophs are not yet innervated by dopaminergic fibres. Measurements of pituitary dopamine levels, as well as immunohistochemical observations of axon terminal density, indicate that the dopaminergic innervation of the IL starts between postnatal day 3 (P3) and P4, and is well developed 8 days later (Davis *et al.* 1984; Gary & Chronwall, 1992).

Melanotropes from adult rats are electrically excitable (Douglas & Taraskevich, 1978; Kehl & McBurney, 1989) and generate a variety of ionic currents in response to

* To whom correspondence should be addressed.

membrane depolarization, including low- and high-threshold Ca^{2+} currents (Cota, 1986; Keja, Stoof & Kits, 1991; Schneggenburger & López-Barneo, 1992). The amplitude of the high-threshold Ca^{2+} current is relatively small in non-dissociated IL cells present in pituitary slices (Schneggenburger & López-Barneo, 1992). It is also small in isolated melanotrophs kept in short-term (~24 h) culture, but increases markedly when the time in culture is prolonged to several days (Cota, 1986; Cota & Hiriart, 1989). This increase can be blocked by culturing the denervated cells in the presence of bromocriptine, a D_2 dopamine agonist, which suggests that Ca^{2+} current expression *in vivo* is also under tonic inhibition by dopamine (Cota & Hiriart, 1989).

In this work we used whole-cell patch-clamp recording to explore, for the first time, the voltage-dependent Ca^{2+} channel activity of rat melanotrophs as a function of postnatal life, from P1 to P20. Our study documents a developmental decrease in melanotroph Ca^{2+} current density that occurs concomitantly with the onset of dopaminergic innervation. In keeping with the previous observations in adult melanotrophs, the developmental fall in Ca^{2+} current expression reflects a decreased activity of high-threshold Ca^{2+} channels and it is likely to result from a long-term dopamine action mediated through D_2 receptors.

A report of some preliminary results has been published in abstract form (Gomora, Navarrete, Marin & Cota, 1993).

METHODS

Animals

Pregnant Wistar rats, bred in our Institute, were housed in individual cages with free access to food and water under a 12 h light, 12 h dark cycle. The day of birth was termed P1. Each litter contained between eight and eleven rats.

Cell preparation and immunocytochemistry

An equal number of male and female rat pups from the same litter, or litters born on the same day, were killed by rapid decapitation at intervals starting on P1. Pituitary glands were removed and separated into anterior lobes and neurointermediate lobes. The anterior lobes were discarded, whereas a pool of six neurointermediate lobes from each age group was incubated for 30 min at 37 °C in minimum essential medium modified for suspension cultures (SMEM; Gibco) supplemented with 0·1% trypsin 1:250 (Difco, Detroit, MI, USA) and 0·1% bovine serum albumin (Sigma). In a few cases, 0·3% collagenase Type 2 (Worthington, Freehold, NJ, USA) was used in place of trypsin. After the enzymatic treatment, the neurointermediate lobes were dissociated into individual cells using Pasteur pipettes with progressively decreasing tip diameters. The monodispersed cells were collected by centrifugation and then diluted in RPMI 1640 medium (Sigma) containing 0·1% bovine serum albumin. Yields increased with postnatal age from approximately 65000 cells per lobe at P2 to about 125000 cells per lobe at P12. Aliquots of the cell suspension were subsequently plated on poly-L-lysine-coated, 10 × 3 mm glass coverslips placed in 35 mm plastic petri dishes. In all cases, the plating density was close to 65000 cells cm^{-2}. The cells were allowed to attach for 60 min in a CO_2 incubator at 37 °C before the addition of standard culture medium, consisting of RPMI 1640 medium supplemented with 5% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. In most experiments, the cells were used within 5–24 h after plating. To examine the effects of prolonged culturing, the cells were maintained in the incubator for up to 8 days and fed with fresh medium every 2 days.

Most (90–95%) of the cultured cells were highly refractile under phase-contrast light microscopy and presented a spherical appearance with diameters ranging from 9 to 13 μm. The cultures also contained some flattened cells, with epithelioid or fibroblast-like morphology. The former corresponded to melanotrophs, as shown by immunocytochemical localization of α-MSH and β-endorphin. These determinations were carried out according to the avidin–biotin peroxidase complex (ABC) method (Childs & Unabia, 1982). In brief, pituitary cells obtained from P2 and P12 rats were treated with B-5 fixative for 10 min. After suppressing endogenous peroxidase activity and non-specific staining, the fixed cells were exposed for 18 h to either rabbit anti-rat β-endorphin serum or rabbit anti-α-MSH serum (Peninsula, Belmont, CA, USA), each diluted 1:2500 in Tris-buffered saline (TBS). Subsequently, biotinylated goat anti-rabbit IgG was applied for 30 min, followed by the ABC Vectastain reagent (Vector, Burlingame, CA, USA) for 45 min. The antibody complex was then developed during a 5 min incubation with 0·05% 3,3′-diaminobenzidine and 0·01% hydrogen peroxide in TBS. More than 93% of the spherical cells were scored as positive for α-MSH and β-endorphin immunoreactivity at both ages examined. In contrast, flattened cells showed no detectable immunostaining.

Drug treatments

The methods for the *in vivo* administration of drugs which are known to interact with D_2 dopamine receptors were similar to those described by Hindelang, Felix, Laurent, Klein & Stoeckel (1990). Briefly, groups of three rat pups per litter were given a daily injection of the active enantiomer of sulpiride (2·5 mg kg^{-1}; Research Biochemicals), bromocriptine (2·5 mg kg^{-1}; Research Biochemicals), or vehicle (6·7 mM tartaric acid and 20% ethanol in saline). Drugs were administered subcutaneously on the animal's back at about 11.00 h. All animals were quickly decapitated 24 h after the third injection and their pituitaries were removed and processed for cell cultures as described above. For *in vitro* treatments, the culture medium was supplemented with a D_2 agonist (1 μM bromocriptine or 1 μM quinpirole; Research Biochemicals), 40 μM actinomycin D or 35 μM cycloheximide (both from Sigma). Control cells were cultured in the presence of 0·04% ethanol. These drugs were applied immediately after cell attachment and were removed 1–3 h before the electrophysiological recording to avoid possible short-term effects on calcium currents (Cota & Hiriart, 1989).

Electrophysiology

Single melanotrophs were subjected to whole-cell patch clamp (Marty & Neher, 1995) at 21 °C using an Axopatch 200A amplifier (Axon Instruments). After removal from the incubator, the cells attached to glass coverslips were placed in a 0·2 ml experimental chamber and then examined within 10–60 min. Initial experiments were conducted using recording solutions of standard ionic composition. The standard external solution contained (mM): NaCl, 145; KCl, 5; $CaCl_2$, 5; Hepes, 10; adjusted to pH 7·3 with NaOH. Patch pipettes pulled from borosilicate glass capillaries were filled with a standard internal solution containing (mM): KCl, 100; NaCl, 30; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 10; Hepes, 10; pH 7·3,

titrated with KOH. For recording currents through Na^+ channels and Ca^{2+} channels in the absence of K^+ current, the cells were bathed in a solution containing (mM): NaCl, 150; $CaCl_2$, 5; Hepes, 10; pH 7·3, and internally dialysed by a pipette solution containing (mM): CsCl, 130; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 10; Hepes 10; buffered to pH 7·3 with CsOH. In subsequent experiments, aimed at recording isolated Ca^{2+} currents after blocking voltage-dependent Na^+ and K^+ channels, the K^+-free internal solution was used in conjunction with a bath solution containing: NaCl, 133 mM; tetraethylammonium chloride, 10 mM; $CaCl_2$, 10 mM; tetrodotoxin (TTX), 1–2 μM; Hepes, 10 mM; pH 7·3. Internal solutions were supplemented with exogenous nucleotides (Na_2ATP, 2 mM and GTP, 50 μM) to prevent changes in Ca^{2+} channel activity caused by prolonged cell dialysis (Cota, 1986). All solutions also contained 5 mM glucose.

Patch electrodes had resistances of 2–3 MΩ and the series resistance was typically in the range 3–6 MΩ. The holding potential was always −80 mV. Cell membrane capacitance was measured as previously described (Meza, Avila, Felix, Gomora & Cota, 1994). Currents were low-pass filtered at 10 kHz with a 4-pole Bessel filter, digitally sampled at intervals of 20 μs and stored on disk using a Labmaster-TL1 interface and pCLAMP software (Axon Instruments). Capacitive transients were electronically minimized and any residual linear component in the resultant current signal was then digitally subtracted on-line using the scaled response to hyperpolarizing voltage steps. In each cell, measurements of voltage-dependent currents at a given membrane potential were made in triplicate and averaged. Current recording was completed within 3 min of establishing contact between pipette and cytosol. Ca^{2+} tail currents were separated into fast and slow components by fitting exponentials to the current signal (Meza *et al.* 1994). Activation curves derived from tail current measurements (see Fig. 2*C*) were fitted by Boltzmann functions: $I = I_{max}/\{1 + \exp[-(V_m - V_{½})/s]\}$, where I is tail current amplitude measured at a constant repolarization level, I_{max} is the saturating value of I after progressively larger activating pulses, V_m is the membrane potential during the test pulse, $V_{½}$ is the voltage for half-maximal activation and s is the

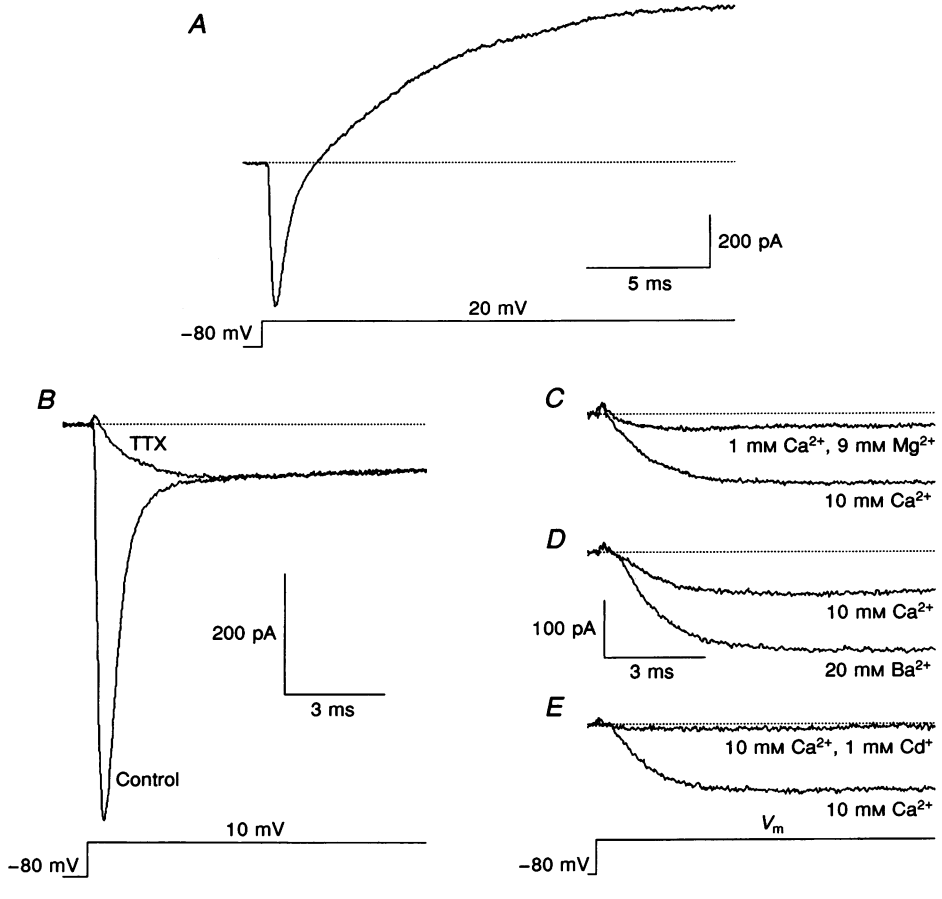

Figure 1. Voltage-dependent ionic currents in neonatal melanotrophs

A, typical pattern of current generated by a P2 cell in response to voltage steps to +20 mV under standard recording conditions. As in all subsequent experiments, the holding potential was −80 mV. Except when indicated otherwise, recordings were obtained from cells cultured for 5–24 h. *B*, inward currents recorded at +10 mV from another P2 melanotroph using K^+-free solutions. Currents were elicited in the absence (Control) or presence of 1 μM external TTX. *C–E*, modulation of the TTX-resistant inward current by changes in the divalent cation composition of the external solution. The current traces were obtained from three different cells at P1 (*C*) or P3 (*D* and *E*). The membrane potential (V_m) during the test pulses was +10 mV (*D*) or +20 mV (*C* and *E*).

slope factor. Data points were subsequently normalized by the corresponding I_{max}. Data are expressed as means ± S.E.M. When appropriate, tests of statistical significance were made using Student's t test or ANOVA.

RESULTS

Ion channel activity of neonatal melanotrophs

Like their adult counterparts, newborn rat melanotrophs kept in short-term culture (5–24 h) express Ca^{2+} channels and other types of voltage-dependent ion channels in the plasma membrane. This characteristic is illustrated in Fig. 1, which presents voltage-clamp experiments performed on P1–P3 melanotrophs. Under standard ionic conditions, the application of 20 ms depolarizing steps to +10 or +20 mV consistently induced a fast, transient inward current followed by a delayed outward current (Fig. 1*A*). The use of K^+-free recording solutions eliminated the outward current and unmasked a second component of inward current that was relatively small and sustained, as shown in Fig. 1*B*. The fast inward current was carried by Na^+ channels since it was blocked by bath application of 1 μM TTX. The second component of inward current was unaffected by TTX and could be ascribed to Ca^{2+} channel activity on the basis of ion substitution experiments. For example, Fig. 1*C–E* shows TTX-resistant inward currents recorded from different cells with 10 mM Ca^{2+} initially present in the bathing solution. As can be seen, the current was greatly reduced after replacing most of external Ca^{2+} by Mg^{2+} (Fig. 1*C*), increased 2-fold in the presence of 20 mM Ba^{2+} (Fig. 1*D*) and was almost completely blocked following the addition of 1 mM Cd^{2+} to the external solution (Fig. 1*E*).

Two major types of voltage-dependent Ca^{2+} channels, named here low- and high-threshold channels, have been identified in melanotrophs from adult rats (Cota, 1986; Stanley & Russell, 1988; Schneggenburger & López-Barneo,

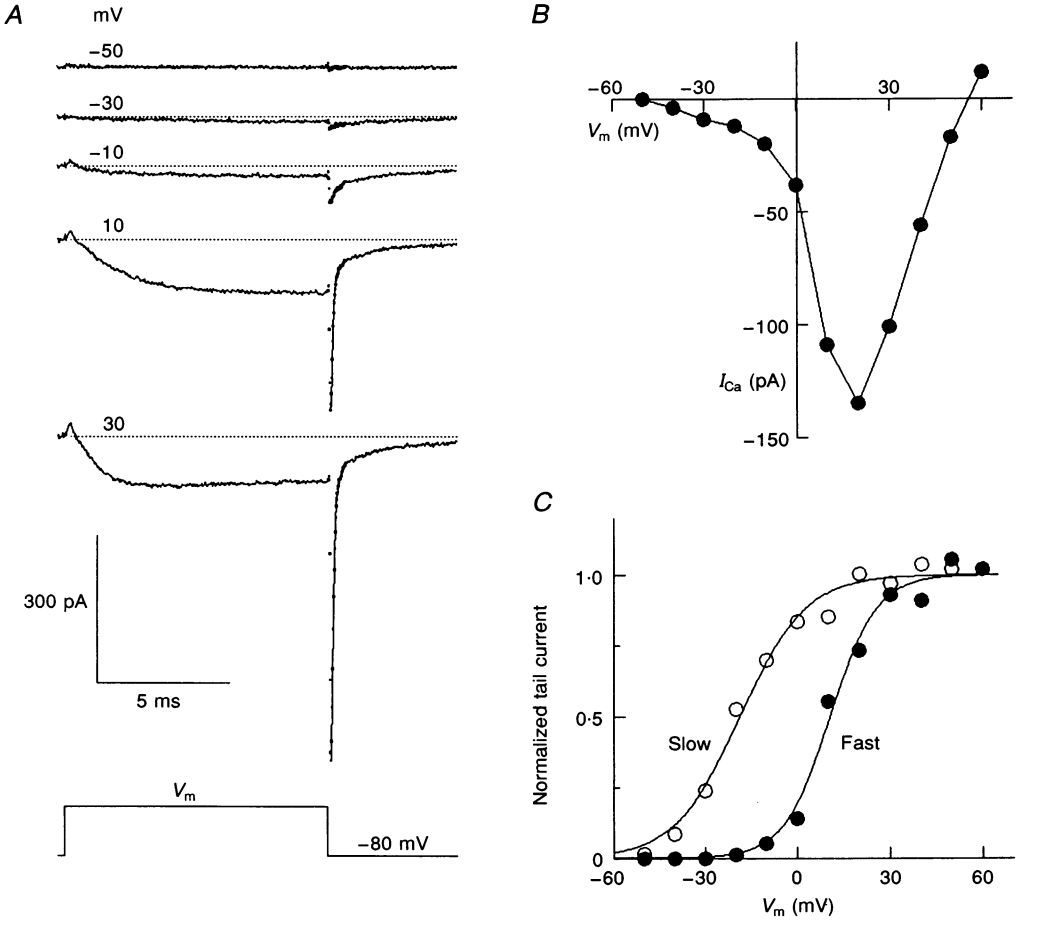

Figure 2. Two components of calcium current

A, Ca^{2+} currents recorded from a P4 melanotroph using 10 ms test pulses to the indicated voltages, followed by repolarization to the holding potential. *B*, dependence of peak Ca^{2+} current (I_{Ca}) on V_m. *C*, normalized tail current amplitude plotted *vs.* V_m to compare the voltage dependence of low- and high-threshold conductance. Data are fitted with smooth lines according to Boltzmann functions. The parameters of the fits, mid-point and slope factor, were −18·7 and 10·65 mV for low-threshold channels (slow tail component) and +10·5 and 7·53 mV for high-threshold channels (fast tail component), respectively.

1992; Kocmur & Zorec, 1993; Keja & Kits, 1994). Among other differences, the low-threshold channels activate over a relatively negative voltage range and close slowly on repolarization, whereas the high-threshold channels require depolarizations above −20 mV for significant activation and deactivate with a fast time course. As illustrated in Fig. 2, the two types of Ca^{2+} channels are already expressed in neonatal melanotrophs. Figure 2*A* shows a family of Ca^{2+} currents induced by 10 ms depolarizations to various membrane potentials and the associated tail currents following repolarization to −80 mV. The traces were obtained from a P4 melanotroph and the corresponding current–voltage (*I–V*) relation is plotted in Fig. 2*B*. At −20 mV or lower depolarizations the Ca^{2+} current was small and flowed mostly through slowly deactivating channels. Depolarizations above −20 mV elicited an additional, larger component of Ca^{2+} current that was carried by fast deactivating channels. To determine the voltage dependence of Ca^{2+} channel conductance, the normalized amplitudes of the fast and slow components in the tail currents were plotted as a function of membrane potential during the preceding activating pulse, as shown in Fig. 2*C*. The resultant conductance–voltage (*G–V*) curves could be well fitted by simple Boltzmann functions. Half of the maximum high-threshold conductance was activated at about +10 mV, whereas the half-maximal activation level for low-threshold channels was close to −19 mV.

Developmental change in calcium current density

To examine whether the Ca^{2+} channel activity of melanotrophs is affected by postnatal development, Ca^{2+} currents were recorded from cells derived from neonatal and immature rats of different ages, from P1 to P20. Pulses to +20 mV were applied from a holding potential of −80 mV in order to induce a near-maximal inward Ca^{2+} current. In addition, capacitance measurements were carried out on each cell investigated to have an index of surface membrane area. The results are summarized in Fig. 3. The peak amplitude of the inward current at +20 mV in P1 melanotrophs was −81 ± 9 pA ($n = 18$). An almost identical value of Ca^{2+} current was obtained at P2, P3 and P4. However, further postnatal development had an

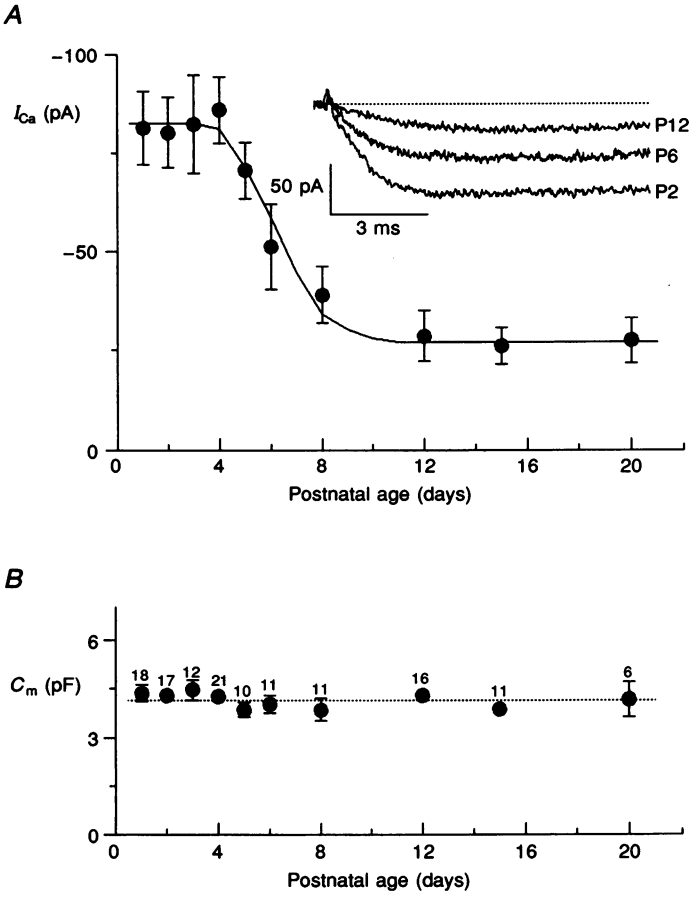

Figure 3. Calcium current density depends on postnatal age

A, summary of measurements of peak Ca^{2+} current at +20 mV (means ± S.E.M.) in melanotrophs of different ages. The number of cells tested within each age group is shown above data points in *B*. The inset shows representative current traces obtained at the indicated postnatal ages. *B*, cell capacitance (C_m) for same cells as in *A*. Regardless of postnatal age, the average value for C_m is close to 4·1 pF (represented by the dashed line).

inhibitory effect as the mean amplitude of the current gradually decreased until P12 when it reached a new stable value (Fig. 3*A*). The new, lower level of Ca^{2+} channel activity corresponded to about one-third of that observed during the first 4 days after birth. This developmental change was not accompanied by any systematic variation in cell capacitance (C_m; Fig. 3*B*), so it mostly reflected a fall in Ca^{2+} current density.

Figure 4 presents current measurements aimed at identifying the type of Ca^{2+} channel affected by postnatal development. The average value for the Ca^{2+} current recorded at −30 mV from P2–P4 melanotrophs was about the same as that in P12–P15 cells, despite the drastic difference in current amplitude at +20 mV between the two groups of cells (Fig. 4*A*). Such a preferential change in the Ca^{2+} current elicited by large depolarizations is confirmed

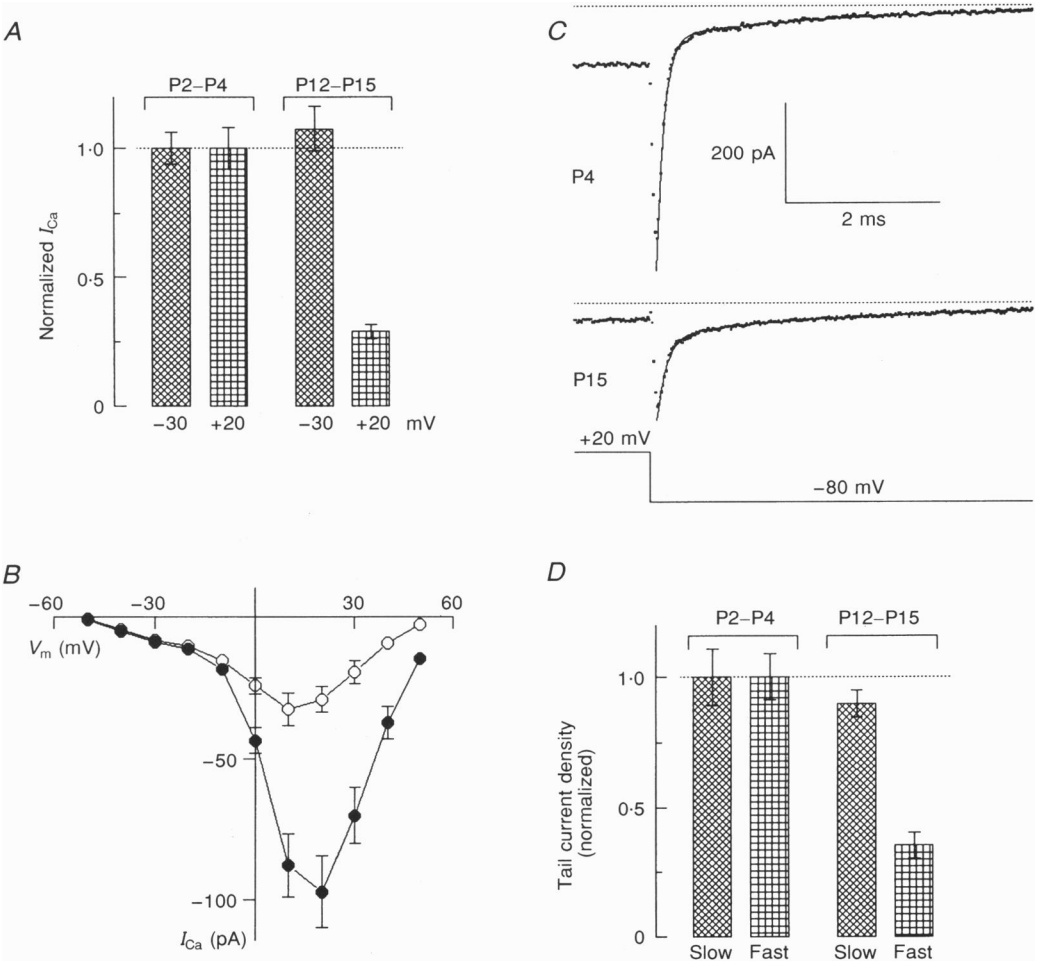

Figure 4. Developmental decrease in the high-threshold component of calcium current

A, comparison of Ca^{2+} current amplitude during 10 ms steps to −30 or +20 mV between two groups of melanotrophs of different ages ($n = 25$ for both). Current amplitude has been normalized relative to the respective mean value in P2–P4 cells (−8·0 ± 0·5 pA at −30 mV and −93·6 ± 7·6 pA at +20 mV). *B*, voltage dependence of inward Ca^{2+} current for melanotrophs at P3 (●) and P12 (○) ($n = 5$ in each case). *C*, Ca^{2+} tail currents recorded at −80 mV from two different melanotrophs after 10 ms activating pulses to +20 mV. Each tail has been fitted by the sum of two exponentials (continuous lines superimposed over data points). In these examples, the time constants of the fitted exponentials were 0·11 and 2·10 ms for the P4 cell, and 0·13 and 2·28 ms for the P15 cell. *D*, normalized amplitudes of the slow and fast tail current components for same cells as in *A*. Ca^{2+} currents were recorded as shown in *C*. In each cell, current amplitude was divided by C_m and then normalized relative to the mean value of current density in P2–P4 cells (−13·7 ± 1·5 and −103·3 ± 9·1 pA pF^{-1} for the slow and fast tail components, respectively). The deactivation time constant for high-threshold channels (fast tail component) was 111 ± 5 μs in P2–P4 cells and 105 ± 5 μs in P12–P15 cells, whereas the deactivation time constant for the low-threshold channels (slow tail component) was 2·33 ± 0·09 and 2·15 ± 0·12 ms, respectively.

in Fig. 4*B*, which shows representative *I–V* curves for inward Ca^{2+} current obtained from P3 and P12 melanotrophs. These curves superimpose almost perfectly in the voltage range between −50 and −20 mV, but greatly differ for membrane potentials more positive than −10 mV. Furthermore, as illustrated in Fig. 4*C* and summarized in Fig. 4*D*, postnatal development did not significantly reduce the amplitude of the slow phase in the tail current recorded after activating steps to +20 mV, but induced a marked decrease in the amplitude of the fast phase.

The developmental suppression of the high-threshold component of Ca^{2+} current occurred without important changes in the voltage dependence of Ca^{2+} channel activation. For example, in the same experiments as in Fig. 4*B*, the mid-point of the *G–V* curve for high-threshold channels was 7·9 ± 1·0 mV in P3 melanotrophs and 6·3 ± 0·7 mV in P12 cells, whereas the slope factor was 7·81 ± 0·59 and 8·48 ± 0·67 mV, respectively. Postnatal development also did not alter the rate of current deactivation at −80 mV (see the legend to Fig. 4*D*).

Role of dopaminergic innervation

The postnatal decrease in melanotroph high-threshold Ca^{2+} channel activity might result from a long-term inhibitory influence of innervation, since it coincides with the development of dopaminergic axons within the IL (Davis *et al.* 1984; Gary & Chronwall, 1992). To test this possibility, we examined the effects of prolonging the time in culture on the Ca^{2+} channel activity of melanotrophs obtained from non- and fully innervated intermediate lobes (P3 and P14, respectively). Figure 5*A* shows peak current amplitude at +20 mV as a function of time in culture. The Ca^{2+} current in denervated P14 cells was small during the first 24 h but then increased 3-fold over the course of the subsequent 4–5 days. By contrast, P3 melanotrophs exhibited a nearly constant Ca^{2+} channel activity throughout the experiment. In both cases, P3 and P14 cells, C_m was practically independent of the age of the culture (Fig. 5*B*). Thus, after a week in culture there was no significant difference in Ca^{2+} current density between the two sets of cells. These results are consistent with the idea that the Ca^{2+} channel activity of P14 melanotrophs, but not that of P3 melanotrophs, is under the control of a long-lasting inhibitory influence *in vivo*.

Figure 6 provides insight into the mechanisms that underlie the increased Ca^{2+} current expression in denervated P14 melanotrophs. In these experiments, some cells were cultured for 5 h under control conditions and

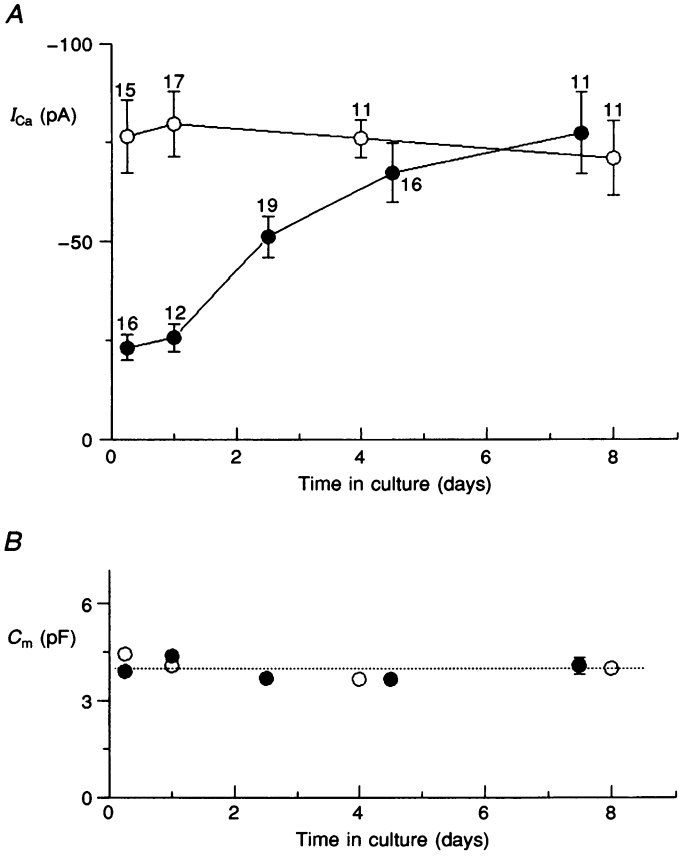

Figure 5. Changes in calcium channel activity induced by prolonged cell culturing
Peak Ca^{2+} current at +20 mV (*A*) and cell capacitance (*B*) for P3 (○) and P14 (●) melanotrophs after different times in culture. The values above each data point in *A* indicate the number of cells investigated.

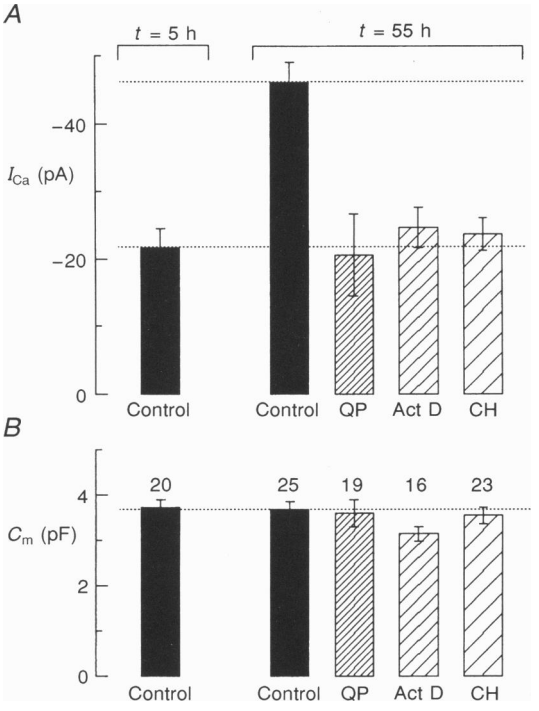

Figure 6. Pharmacological blockage of calcium current expression in cultured melanotrophs

A, peak Ca^{2+} current recorded at +20 mV from several groups of P14 melanotrophs. Before these determinations, the cells were cultured for the indicated times (*t*) under control conditions or in the presence of quinpirole (QP; 1 μM), actinomycin D (Act D; 40 μM) or cycloheximide (CH; 35 μM). *B*, corresponding cell capacitance values. The number of cells examined is given above each bar.

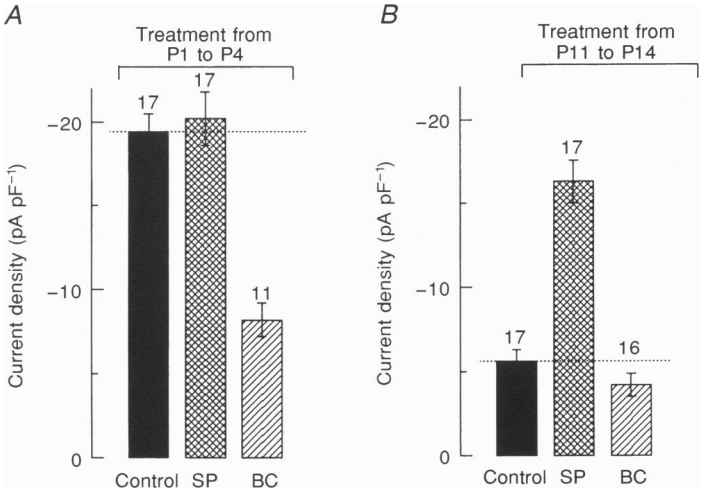

Figure 7. Regulation of calcium channel activity by chronic *in vivo* treatment with dopaminergic compounds

Ca^{2+} current density at +20 mV in melanotrophs obtained from P4 (*A*) and P14 (*B*) rats that had been treated for 3 days with sulpiride (SP; 2·5 mg kg^{-1}), bromocriptine (BC; 2·5 mg kg^{-1}) or vehicle (Control).

then subjected to patch-clamp recording. The average value for peak inward current at +20 mV in these cells was approximately −22 pA (Fig. 6*A*, bar to the left). Other groups of P14 melanotrophs were cultured for about 55 h either under control conditions or in the presence of standard medium supplemented with quinpirole, bromocriptine, actinomycin D or cycloheximide. Quinpirole, like bromocriptine, is a selective D_2 dopamine receptor agonist (Gingrich & Caron, 1993), whereas actinomycin D and cycloheximide are well-known inhibitors of gene transcription and protein synthesis, respectively. As already shown in Fig. 5*A*, prolonging the time in culture from 5 to 55 h induced a 2-fold increase in Ca^{2+} current amplitude in control cells. This effect was completely blocked by the continuous presence of quinpirole in the culture medium (Fig. 6*A*, bars to the right). Similar results were obtained with the application of bromocriptine (data not shown). Furthermore, the inhibitory action of the dopamine agonists was mimicked by actinomycin D and cycloheximide. Surface membrane area, on the other hand, was in general unaffected by these drugs. The only exception was actinomycin D, which caused a 15% reduction in C_m relative to control cells (Fig. 6*B*). Therefore, the increase in Ca^{2+} channel activity of previously innervated melanotrophs with time in culture involves *de novo* synthesis of both RNA and protein, and seems to be triggered by the loss of a tonic dopaminergic input.

To further test the hypothesis that D_2 receptor stimulation plays a central role in the developmental regulation of melanotroph Ca^{2+} channel activity, we treated rats with dopaminergic agents. Newborn and immature rats (P1 and P11, respectively) received daily subcutaneous injections of the D_2 receptor antagonist sulpiride or bromocriptine, and were killed 24 h after the third treatment (i.e. on P4 and P14). Melanotropes obtained from control and drug-treated pups were electrophysiologically examined within 5–24 h to determine Ca^{2+} current density at +20 mV. Figure 7 summarizes the results of these *in vivo* pharmacological manipulations. Sulpiride had no significant effect on Ca^{2+} channel activity when administered prior to the onset of dopaminergic innervation (Fig. 7*A*) but induced a 3-fold increase in current density, compared with the respective control value, once innervation was fully developed (Fig. 7*B*). Bromocriptine treatment, on the other hand, depressed Ca^{2+} channel activity at both ages examined. This effect was marked on P4 (57% reduction in current density; Fig. 7*A*) and less pronounced after innervation (25% reduction; Fig. 7*B*). Thus, the developmental suppression of Ca^{2+} channel activity can be mimicked or reversed by chronic *in vivo* treatment with a D_2 agonist or antagonist, respectively.

DISCUSSION

In this study, we show that Ca^{2+} current density through high-threshold Ca^{2+} channels in rat pituitary melanotrophs undergoes a striking reduction during the early postnatal period. Additionally, we provide evidence that such a developmental change in ion channel activity results from the innervation of melanotrophs by dopaminergic neurons, and that it is mediated by chronic stimulation of D_2 dopamine receptors.

The decrease in Ca^{2+} current density occurs between P4 and P12, a period that coincides with the development of dopaminergic axons and increased dopamine levels within the IL as demonstrated by previous authors (Davis *et al.* 1984; Gary & Chronwall, 1992). This temporal correlation suggests a critical role for dopamine and D_2 receptor activation in the developmental regulation of melanotroph Ca^{2+} channel activity. Consistent with this possibility, we found that before the onset of innervation (P4), Ca^{2+} current density can be decreased by chronic *in vivo* treatment with the D_2 agonist bromocriptine; conversely, after the onset and full development of innervation (P14), current density can be increased by *in vivo* administration of the D_2 antagonist sulpiride. Furthermore, Ca^{2+} current density in denervated P14 melanotrophs kept in culture for several days increases spontaneously as a function of time, until reaching a high level similar to that normally exhibited by P3 melanotrophs; this increase is not seen in P14 cells cultured in the presence of D_2 agonists.

A stimulatory effect of denervation followed by prolonged cell culturing on melanotroph high-threshold Ca^{2+} channel activity was initially reported by Cota (1986) using adult rat IL cells. Subsequent experiments showed that this effect can be prevented or even reversed by chronic exposure of the cultured cells to bromocriptine (Cota & Hiriart, 1989). More recently, a similar time-dependent increase in Ca^{2+} channel activity, and its block by continuous treatment with D_2 receptor agonists, has been inferred from measurements of the intracellular Ca^{2+} response to K^+ depolarization in adult rat IL lobules maintained under tissue culture conditions (Chronwall, Beatty, Sharma & Morris, 1995). The experiments presented here extend these findings to immature rat melanotrophs and, moreover, indicate that a long-term dopamine action mediated through D_2 receptors underlies the reported postnatal change in melanotroph Ca^{2+} channel activity.

Our Ca^{2+} current measurements were made at least 5 h after cell dissociation and plating and thus reveal the persistent effects of postnatal development and D_2 receptor activation on voltage-dependent Ca^{2+} channel activity. Further experiments will be needed to determine whether

D_2 receptors in neonatal melanotrophs are able to mediate additional short-term modulatory effects on Ca^{2+} current density, as those described in melanotrophs from adult rats (Cota & Hiriart, 1989; Williams, MacVicar & Pittman, 1990; Stack & Surprenant, 1991; Keja, Stoof & Kits, 1992) and frogs (Valentijn, Vaudry & Cazin, 1993).

Long-term effects of intercellular chemical messengers on Ca^{2+} current density have also been observed in the GH_3 pituitary cell line and they seem to involve regulation of Ca^{2+} channel expression (Ritchie, 1993; Meza *et al.* 1994). Likewise, the developmental suppression of melanotroph Ca^{2+} channel activity may be due to a decrease in the number of functional Ca^{2+} channels in the plasma membrane. This hypothesis is supported by the fact that the onset of dopaminergic innervation is not accompanied by any significant change in the voltage dependence of Ca^{2+} channel activation or channel deactivation rate. Furthermore, recovery from the inhibitory influence of innervation, that is the growth of Ca^{2+} current after melanotroph denervation and prolonged culturing, requires new RNA and protein synthesis, as it is blocked not only by D_2 agonists but also by actinomycin D and cycloheximide. Thus, chronic D_2 receptor activation could depress Ca^{2+} current density by decreasing the rate of Ca^{2+} channel biosynthesis. Alternatively, stimulating the D_2 receptors may inhibit the expression of regulatory proteins that control the fraction of functional Ca^{2+} channels.

A general property of D_2-like receptors is that they are negatively coupled to adenylate cyclase (Cote, Grewe, Tsuruta, Stoof, Eskay & Kebabian, 1982; Gingrich & Caron, 1993). Since treatment of adult melanotrophs in culture with a permeable analogue of cAMP, 8-bromo-cAMP, prevents the inhibition of Ca^{2+} channel activity induced by chronic application of bromocriptine (Cota & Hiriart, 1989), it can be further suggested that the developmental change in melanotroph Ca^{2+} current density results from a decrease in the intracellular concentrations of cAMP. A precedent for this view is that cAMP regulates Na^+ channel biosynthesis in developing rat muscle cells (Offord & Catterall, 1989). On the other hand, D_2 receptor antagonists have been shown to increase the expression of the immediate early gene c-*fos* in enkephalin-containing mammalian striatal neurons (Robertson, Vincent & Fibiger, 1992). Along this regard, Chronwall *et al.* (1995) have found that the addition of c-*fos* antisense oligodeoxynucleotide to the culture medium mimics the depressive effect of chronic D_2 agonist treatment on adult melanotroph Ca^{2+} channel activity. It will therefore be of interest to investigate whether the activation of D_2 receptors controls Ca^{2+} current expression in neonatal melanotrophs through a signalling pathway involving decreased production of cAMP and subsequent reduction of Fos levels.

Regardless of its molecular basis, the developmental change in Ca^{2+} channel activity we have observed is likely to have functional implications. Calcium influx through voltage-gated Ca^{2+} channels promotes the exocytotic release of melanotroph peptides (Thomas, Suprenant & Almers, 1990) and also contributes to maintain POMC synthesis (Loeffler, Kley, Pittius & Höllt, 1986). Consequently, a drop in Ca^{2+} current density should lead to a decrease in the biosynthetic and secretory activity of the melanotrophs. In agreement with this expectation, α-MSH serum concentrations drastically decrease between P6 and P14 (Davis *et al.* 1984). Moreover, chronic *in vivo* administration of the dopamine antagonist haloperidol significantly enhances POMC gene expression in the rat IL beginning 5 days after birth (Hindelang *et al.* 1990). Interestingly, the dopaminergic innervation also regulates melanotroph proliferation (Chronwall *et al.* 1987; Gary & Chronwall, 1992). Whether Ca^{2+} channel activity plays a role in the control of cell cycle progression within the IL, as it does in cultures of clonal pituitary cells (Ramsdell, 1991), remains to be determined.

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