

# Differential Involvement of $\text{Ca}^{2+}$ Channels in Survival and Neurite Outgrowth of Cultured Embryonic Cockroach Brain Neurons

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**Benquet, Pascal, Janine Le Guen, Yves Pichon, and François Tiaho.** Differential involvement of  $\text{Ca}^{2+}$  channels in survival and neurite outgrowth of cultured embryonic cockroach brain neurons. *J Neurophysiol* 88: 1475–1490, 2002; 10.1152/jn.00749.2001. The contribution of voltage-gated calcium channels (VGCC) to the development of cultured embryonic cockroach brain neurons was assessed using pharmacological agents. VGCC currents were recorded using the patch-clamp technique and were found to be blocked dose-dependently by micromolar concentrations of mibefradil. The activation and inactivation properties of the calcium channels enable a sizeable calcium current to flow at rest (about  $-30$  and  $-20$  mV in high-potassium culture media). As expected, the cytoplasmic-free calcium concentration was found to rise when the extracellular potassium concentration was raised from 3 to 15 and 30 mM. The effects of VGCC blockers and calcium chelators were different in fresh and in mature cultures in which the neurons were connected to each other to form a defined network. In fresh cultures, the two non-selective VGCC blockers (verapamil and mibefradil) induced a dose-dependent cell death that was proportional to their blocking effect on  $I_{\text{Ba}}$ . This effect could not be prevented by addition of fetal calf serum to the culture medium. A similar effect was obtained using intra- or extracellular calcium chelating agents (10  $\mu\text{M}$  BAPTA-AM or 10 mM EGTA). Quite unexpectedly, blockade of the P/Q-like ( $\omega$ -Aga WA-sensitive) component of the calcium current by 500 nM of  $\omega$ -AgaTx IVA had no lethal effect, suggesting that the corresponding channels are not involved in the survival mechanism. As expected from their lack of effect on  $I_{\text{Ba}}$ , isradipine, nifedipine, and  $\omega$ -CgTx GVIA did not induce cell death. When the neurons started growing neurites, their sensitivity to calcium channel blockade by mibefradil decreased, indicating a correlation between neurite outgrowth and resistance to calcium depletion. In mature cultures, the neurons became resistant to mibefradil, verapamil, and BAPTA-AM. However, these agents, as well as  $\omega$ -AgaTx IVA, had a significant inhibitory effect on the increase in diameter of the connectives that linked adjacent clusters of neurons. This effect has been shown to result, in the case of mibefradil, from an inhibition of neurite outgrowth characterized by a significant reduction of the number of primary neurites and secondary branchings but not to a significant modification of the diameter of individual neurites. These results support the view that, as in vertebrates, calcium influx through VGCC plays an important role in survival and neurite outgrowth of cultured embryonic insect neurons. The differential contribution of the P/Q-like and R-like ( $\omega$ -Aga WA-sensitive) calcium channels in these processes is discussed.

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## INTRODUCTION

In the CNS of vertebrates, voltage-gated calcium channels (VGCC) are known to be implicated in synaptic plasticity and gene transcription in addition to their “classical” role in neurotransmission and regulation of cell excitability (Berridge 1998; Bito et al. 1997; Finkbeiner and Greenberg 1998). In vitro, in the absence of neurotrophic factors, neuron survival is rescued by activation of calcium influx through L-type VGCC (Collins and Lile 1989; Gallo et al. 1987; Koike et al. 1989; Murrell and Tolkovsky 1993; Yano et al. 1998). It is only recently that a tyrosine kinase (Trk)-like receptor has been identified in the CNS of invertebrates (Lucini et al. 1999; van Kesteren et al. 1998). The difficulties in identifying neurotrophins has impeded studies about the mechanisms of neuron survival and growth in invertebrates. Despite the observation that survival and growth of insect cultured neurons needed a culture medium with high  $\text{K}^+$  (Beadle and Hicks 1985), suggesting that depolarization of cell membrane might be a prerequisite, little is known about the role of VGCC, and therefore calcium influx, on neuronal survival and neurite outgrowth in insects.

So far, most VGCC found on insect neuron somata were found to be insensitive to dihydropyridine (DHP) and sensitive to both  $\omega$ -CgTx GVIA or  $\omega$ -AgaTx IVA toxin fractions (Benquet et al. 1999; Bickmeyer et al. 1994; Wicher and Penzlin 1997). They have also been shown to be functional early in neuronal development (Baines and Bate 1998; Goodman and Spitzer 1979), a finding that is consistent with the hypothesis that they might, as in vertebrates, play a key role in different aspects of neuronal differentiation such as survival or neurite outgrowth. This hypothesis is also consistent with the observations that, in *Drosophila*, null mutation of the genes coding for VGCC (Smith et al. 1996) or mutations reducing the activity of the calcium/calmodulin-dependent protein kinase-II (Griffith 1997) are lethal during embryogenesis.

We have previously shown that (Benquet et al. 1999) 1) embryonic cockroach brain neurons in primary culture express at least two type of high voltage activated (HVA) calcium channel currents named P/Q-like (sensitive to  $\omega$ -AgaTx IVA) and R-like (insensitive to DHP and the 3 toxins  $\omega$ -CgTx GVIA,  $\omega$ -CmTx MVIIC, and  $\omega$ -AgaTx IVA) and 2) the phenylalkylamine (PAA) verapamil is a non-selective blocker since it

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suppressed efficiently both components of the calcium channel current. To directly assess the physiological role of VGCC in neuronal development in insects, we have analyzed the effects of selective and non-selective blockers using the whole cell configuration of the patch-clamp technique and compared these effects with those of these same blockers on survival and neurite outgrowth.

Our experiments indicate that, as in vertebrates, VGCC are involved in neuronal survival and neurite outgrowth. Furthermore, the results suggest that the activation of the DHP-,  $\omega$ -CgTx GVIA-,  $\omega$ -CmTx MVIIC-, and  $\omega$ -AgaTx IVA-resistant current components (R-like) is important for neuronal survival, whereas the  $\omega$ -AgaTx IVA-sensitive current component (P/Q-like) is selectively involved in neurite outgrowth.

## METHODS

### Cell culture

The culture technique was derived from that of Chen and Levi-Montalcini (1970), as described by Beadle and Hicks (1985) and recently modified (Angevin et al. 2000). Briefly, newly laid eggs were stored for 21–23 days in an incubator (28–29°C). The heads of the embryos were removed from the egg cases, and the brains dissected out by removing the head capsule. The cells were dissociated mechanically by gentle mechanical trituration with a Pasteur pipette in a defined volume of culture medium. The cultures were initiated in a medium (5 + 4) containing five parts of Schneider's revised *Drosophila* medium and four parts of Eagle's basal medium containing 100 i.u./ml penicillin and 100  $\mu$ g/ml streptomycin complemented with 6 mg/ml L-glutamine and 2.5  $\mu$ g/ml fungizone. After 5 days, the first (5 + 4) culture medium was replaced by a second (L + G) medium made of equal parts of Leibovitz's L-15 medium and Yunker's modified Grace medium containing penicillin, streptomycin, glutamine, and fungizone, supplemented with 10% fetal calf serum. It should be noted that both culture media contained high K<sup>+</sup> concentrations (16 mM in the former, 30 mM in the latter), which favored the development of the cultures. The Ca<sup>2+</sup> concentrations were 4 mM in the first medium and 3 mM in the second medium. Low intra- or extracellular calcium concentrations were obtained by adding 10  $\mu$ M BAPTA-AM (30–60 min) or 10 mM EGTA to the culture media. The resulting free calcium concentration was estimated to be lower than 25 and 75 nM, respectively, inside and outside the cells, assuming  $K_d$  values of, respectively, 0.2 and 0.07  $\mu$ M for the binding of calcium to the two chelators. The culture media were obtained from GIBCO-BRL (Cergy Pontoise, France).

### Electrophysiology

Currents flowing through the calcium channels were studied using the whole cell configuration of the patch-clamp technique (Benquet et al. 1999; Christensen et al. 1988; Hamill et al. 1981). Whenever necessary, the culture medium was replaced with a recording solution containing (in mM) 100 tetraethylammonium (TEA)Cl, 70 Tris-HCl, 10 4-AP, 10 BaCl<sub>2</sub>, 4 MgCl<sub>2</sub>, and 10 HEPES buffered at pH 7.2 using TEA-OH. Unless noted otherwise, the patch electrodes were filled with a solution containing (in mM) 120 CsF, 25 CsOH, 2 MgCl<sub>2</sub>, 10 EGTA, 3 ATP-Mg<sup>2+</sup>, 0.5 GTP-Tris, and 10 HEPES buffered at pH 7.3 using CsOH. The resistance of these electrodes ranged from 2 to 5 M $\Omega$ . Voltage-clamp experiments were performed with the patch-clamp amplifier RK300 (Biologic Science Instrument, Claix, France). Unless stated otherwise, the holding potential (HP) was -70 mV. All experiments were performed at room temperature (20–27°C). The pClamp (Axon Instruments) 5.5 software was used for stimulation, data acquisition, and analysis. Whenever necessary, the data were analyzed off-line using

different software packages: Excel (Microsoft), and Sigmaplot (Jandel Scientific). The Student's *t*-test was used for statistical analysis.

The peak current-voltage relationships (*I-V* curve) were fitted with the following Boltzmann equation

$$I = G_{\max} \times (V - E_{\text{Ba}}) / \{1 + \exp[(V_{0.5} - V)/K]\} \quad (1)$$

where  $G_{\max}$  is the maximal conductance of the global calcium channels,  $E_{\text{Ba}}$  is the estimated reversal potential of  $I_{\text{Ba}}$ ,  $V_{0.5}$  is the potential for half-maximal steady-state activation of the barium current, and  $K$  is a voltage-dependent slope factor. The dose-response curve were fitted with a Hill equation

$$I/I_{\max} = 100 \times [X]^n / (IC_{50}^n + [X]^n) \quad (2)$$

where  $I_{\max}$  and  $I$  are the peak current amplitudes triggered by test depolarizations from -70 mV (HP) to 0 or +10 mV, respectively, in the absence and the presence of a range of concentrations ( $[X]$ ) of the calcium channel blockers,  $n$  is the Hill coefficient, and  $IC_{50}$  the concentration needed to produce 50% inhibition.

### Optical measurements of high K<sup>+</sup>-induced changes in intracellular calcium

Changes in intracellular calcium induced by high-K<sup>+</sup> solutions were monitored using the calcium fluorescent probe Fura-2, according to the technique described by Eilers et al. (1995), Grynkiewicz et al. (1985), and Kirischuk and Verkhratsky (1996). Briefly, neurons were loaded with the acetoxymethyl ester of Fura-2 (4  $\mu$ M), during 30 min in the culture medium at room temperature (20–27°C). Intracellular calcium was measured by microscopic photometry using an Olympus IMT-2 inverted microscope equipped with a 40 $\times$  lens (Olympus apo40UV oil). The illumination unit (a XBO 75 W Osram xenon arc lamp) and a dual wavelength (352 and 380 nm) excitation monochromator were connected to the microscope via a quartz fiber optic bundle, and the excitation light was reflected toward the culture dish by a 400-nm dichroic mirror. The emitted fluorescence produced after excitation was filtered through a long wave-pass filter with a 510 nm barrier. The excitation-induced fluorescence of the few cells of a previously selected region of interest was detected by a photomultiplier (710 PMT; Photon Technology Int.) connected to the microscope (via a photometer D-104B; Photon Technology Int.) through the side camera port of the microscope. The fluorescence data were analyzed on a PC computer using the adequate software (Felix; Photon Technology Int.), and the variations of the intracellular calcium levels were computed from the ratios of the fluorescence at the two wavelengths (F352/F380) after subtraction of the background fluorescence. The standard saline had the following ionic composition (in mM): 205 NaCl, 3.1 KCl, 5 CaCl<sub>2</sub>, 4 MgCl<sub>2</sub>, and 10 HEPES, and its pH was adjusted to 7.4 using NaOH. The high K<sup>+</sup> solutions contained respectively 15 (K15) and 30 (K30) mM KCl. The NaCl concentrations of these two solutions were reduced to 193 and 178 mM, respectively.

### Morphological analysis

GENERAL ASSESSMENT OF SURVIVAL AND GROWTH. The effects of the pharmacological treatment on survival and growth were studied using an inverted microscope (OLYMPUS) connected to 486 PC clone via a COHU solid State CCD camera. The average number of living neurons, clusters, and diameters of the fiber bundles was estimated from 12 to 16 fields (0.5 mm<sup>2</sup> each) selected to reflect the entire culture as faithfully as possible. The selection was done as follows: starting from its left edge, the culture was scanned horizontally and every third field examined until the right edge was reached. The culture was then scanned vertically using the same procedure from its upper to its lower edge. This method was found to minimize the errors resulting from the uneven distribution of the neurons in the culture dish. For each experiment, a minimum of four culture dishes

was used:  $\geq 2$  for the tests and  $\geq 2$  for the controls (which originated from the same culture batch as the treated neurons and were treated in the same way apart from the addition of the test molecule).

Neuron survival was assessed from the brightness of the living neurons under phase contrast and their inability to be labeled with trypan blue (20%). To quantify neurite outgrowth, digitized video images of cultures were used for each field and the diameter of the largest nerve bundle (which has been shown to reflect neurite outgrowth, see RESULTS) was measured with OPTIMAS software. For each experiment, SE was computed from the values of each individual field (i.e.,  $\geq 24$  fields). Whenever necessary, the number of analyzed field (nf) is indicated. All experiments were reproduced at least three times before their validation. Data were analyzed using the Student's *t*-test. The *P* values are illustrated as follows: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

### Detailed morphological analysis

To resolve and quantify the fine structure of individual neurons, Lucifer yellow (1–5 mg/ml) was added to the pipette solution and allowed to diffuse inside the cell through the patch pipette after the establishment of the whole cell configuration. After a 10-min perfusion, the patch pipette was withdrawn and the morphology of the perfused neuron examined under an epifluorescence microscope and photographs taken. Measurements of the length of the neurites and of their ramification pattern were performed on these photographs.

Scanning electron microscopy has been used to visualize the possible effects of calcium channel blockers on the fine morphology of the dendrites (which is not accessible with the conventional optical microscopy). The cultures were fixed using 2.5% glutaraldehyde in 0.2 M phosphate buffer at pH 7.3, and rinsed four times for 5 min in 0.1 M phosphate buffer. The buffer was replaced by a 70% ethanol solution for 10 min and then 95% ethanol for 20 min, and the preparation stored in absolute ethanol. The bottom of the culture dish (on which the cultured neurons adhered) was cut and transferred into a tight-sealed chamber enabling progressive replacement of the alcohol by liquid carbon dioxide. The temperature was raised to the critical point (about 35°C) and the pressure slowly dropped. The dry culture samples were coated with metal before examination with the scanning electron microscope.

### Pharmacological agents

Verapamil, isradipine, nifedipine, BAPTA-AM, and EGTA were purchased from Sigma Chemical (Isle d'Abeau Chesnes, France). Mibefradil (Hoffman Laroche) and verapamil solutions were prepared extemporarily. Isradipine and nifedipine were dissolved in DMSO to obtain a stock solution (10 mM) that was further diluted before use. The final DMSO concentrations never exceeded 0.1%, the concentration that was found to have no detectable effect on  $I_{Ba}$ , neuron survival, or neurite outgrowth.  $\omega$ -CgTx GVIA (Sigma) and  $\omega$ -AgaTx IVA (Calbiochem) were dissolved in distilled water at  $10^{-4}$ – $10^{-3}$  M and stored at  $-70^{\circ}\text{C}$ .

## RESULTS

### Block of the voltage-gated calcium channel current by mibefradil

The goal of this study was to evaluate the role of VGCC in the *in vitro* development of cockroach brain neurons. To achieve this goal, we needed a set of specific blockers of these channels. We have shown earlier (Benquet et al. 1999) that  $\omega$ -AgaTx IVA was a potent but partial blocker and that the phenylalkylamine verapamil could reversibly block all the current but at concentrations (more than 1 mM) that were

probably not selective for VGCC. We have therefore considered using mibefradil as an alternative for verapamil. This molecule was originally considered to be selective for low-voltage-activated calcium channels (Osterrieder and Holck 1989) but has been later found to also block high-voltage-activated VGCC on vertebrate preparations (Bezprozvanny and Tsien 1995; Viana et al. 1997) as well as in dissociated embryonic cockroach neurons (Benquet et al. 2000). Bath superfusion of these insect neurons with mibefradil concentrations ranging from 0.1 to 10  $\mu\text{M}$  blocked  $I_{Ba}$  in a dose-dependent manner (Fig. 1, *A* and *B*). A significant reduction of the current was observed for 1  $\mu\text{M}$ , and 10  $\mu\text{M}$  reduced the current by about 90%. 100  $\mu\text{M}$  mibefradil completely blocked the barium current in 5-day-old ( $n = 5$ ) and 16-day-old ( $n = 4$ ) cultures. The blocking kinetics were fast. Thus with 10  $\mu\text{M}$ , the steady state was reached within 1 min (Fig. 1*B*). This inhibition of the current was partly reversible after return to a drug-free solution (Fig. 1*B*) and was voltage independent as illustrated in Fig. 1*C* for five potential levels. The dose-response relationship established under steady-state conditions (i.e., after incubation of the neurons for  $\geq 10$  min in the presence of different concentrations of the blocking molecule) yielded an  $\text{IC}_{50}$  of 1.5  $\mu\text{M}$  and a Hill coefficient of  $n = 1.1$  when fitted with the Hill equation (Fig. 1*D*). Mibefradil was therefore about 100 times more potent than verapamil on our preparation and has consequently been used in most experiments described in this paper.

### Evidence that VGCC can be activated and remain activated in the high- $\text{K}^+$ culture media

Under our culture conditions neurons are likely to be depolarized by the high- $\text{K}^+$  concentration of the culture media (16 mM in the first medium and 30 mM in the second, see METHODS). To estimate the resting potential of the neurons, we have replaced CsF and CsOH in the pipette solution by equimolar concentrations of K-gluconate and KOH and measured the resting potential (RP) of these neurons maintained in the culture media (using the current-clamp mode of the patch-clamp technique). The average RP was  $-27 \pm 4$  mV ( $n = 7$ ) in the first medium and  $-19 \pm 1$  mV ( $n = 19$ ) in the second. Similar RP values,  $-29 \pm 2$  mV ( $n = 13$ ) and  $-21 \pm 1$  mV ( $n = 9$ ), were found if the culture media were replaced by extracellular solutions containing the same  $\text{K}^+$  concentrations as the culture media (16 or 30 mM) but in which TEACl was replaced by an equimolar concentration of NaCl (see METHODS). In a previous paper on this same preparation (Benquet et al. 1999), we had shown that, for these membrane potential values, the barium current was activated (activation threshold at about  $-30$  mV) and was only partly inactivated (potential of half-inactivation at  $-30$  mV), suggesting that, at the above resting potential levels, a significant current might flow through the calcium channels. One could not exclude, however, that long-term depolarization could inactivate the current. We have therefore tested this possibility and recorded calcium currents ( $I_{Ca}$ ) from neurons that had been maintained in the most depolarizing medium (L + G:  $\text{K}^+ = 30$  mM;  $\text{Ca}^{2+} = 3$  mM) at holding potentials close to the estimated RP of these neurons ( $-30$  and  $-20$  mV). Under these conditions, membrane depolarization to 0 mV from a HP of  $-30$  mV, or 10 mV from a HP of  $-20$  mV, induced a calcium current (Fig. 2*A*, *a* and *b*) averaging  $-87 \pm 15$  pA ( $n = 13$ ) and  $-33 \pm 10$  pA ( $n = 5$ ), respectively. This



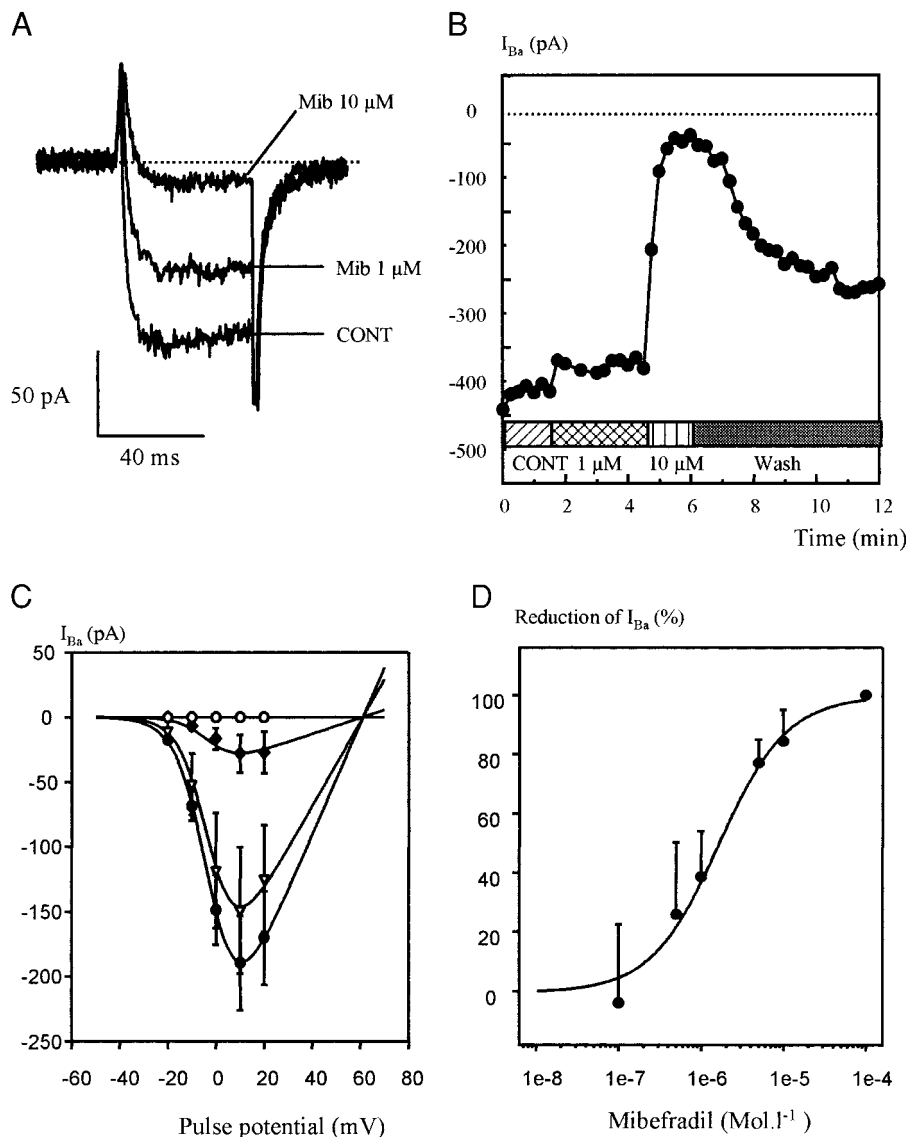


FIG. 1. Dose-dependent effect of mibefradil on the barium currents ( $I_{Ba}$ ) recorded from the soma of an embryonic cockroach brain neuron in primary culture with 10 mM  $Ba^{2+}$  in the bathing medium. Seven-day-old culture. *A*: typical current traces obtained at steady state and illustrating the dose-dependent reduction of  $I_{Ba}$  on bath superfusion of the neuron without (CONT) and with 1 or 10  $\mu$ M mibefradil (Mib) as indicated.  $I_{Ba}$  was elicited by a voltage pulse to 0 mV from a holding potential (HP) of -70 mV. The horizontal dotted line indicates the 0 current level. No leak and capacitance subtraction was done. *B*: kinetics of the effects of 2 concentrations of mibefradil (as indicated) on the peak amplitude of the barium current ( $I_{Ba}$ ). Note that the effects were fast and partially reversible after return to drug-free saline (wash). *C*: steady-state current-voltage ( $I$ - $V$ ) relationship of the average peak amplitude of  $I_{Ba}$  recorded without (●) or in the presence of 0.5  $\mu$ M (▽), 5  $\mu$ M (◆), and 100  $\mu$ M (○) mibefradil. Neurons were incubated in the test solution for  $\geq 10$  min before measuring the peak amplitude of  $I_{Ba}$  (from the 0 current level). Each data point was obtained from  $\geq 7$  neurons from 2 different cultures. The vertical bars represent  $\pm$ SE. The smooth curves represents the fit of the experimental points with Boltzmann equation 1: ( $I = G_{max} \times (V - E_{Ba}) / (1 + \exp[(V_{0.5} - V)/K])$ ) with  $E_{rev} = 61 \pm 1$  mV,  $V_{0.5} = -2 \pm 1$  mV,  $K = 6 \pm 1$  for all 4 curves, and  $G = 4.2 \pm 1.0$  nS (control),  $3.2 \pm 1.0$  nS (0.5  $\mu$ M),  $0.6 \pm 2.0$  nS (5  $\mu$ M), and  $0 \pm 0$  nS (100  $\mu$ M). *D*: dose-response curve representing the percentage of reduction of the peak current by mibefradil concentrations ranging from 0.1 to 100  $\mu$ M. The data points (●) represent the mean reduction of  $I_{Ba}$  from 6 to 10 neurons from 5 different cultures  $\pm$ SE (vertical bar). The smooth line represents the fit of the data points with the Hill equation (see Eq. 2 in METHODS) with an  $IC_{50} = 1.5$   $\mu$ M and a Hill coefficient ( $n$ ) of 1.1.

current, which was blocked by cadmium (data not shown) and mibefradil (Fig. 2Aa, inset), exhibited slower activation and deactivation kinetics than those observed under standard conditions. For a depolarization to 0 mV, the time to peak was  $68 \pm 9$  ms ( $n = 9$ ) compared with about 10 times less (5.7 ms) under normal conditions (Benquet et al. 1999), whereas the deactivation from 0 to -30 mV was bi-exponential with fast and slow time constants of  $5 \pm 2$  ms and  $42 \pm 9$  ms ( $n = 8$ ), respectively, against a full deactivation time of  $< 3$  ms under normal conditions (Benquet et al. 1999). The mechanism underlying this phenomenon, which was fully reversible when the external culture media was replaced with normal saline, remains to be studied. At these HP, a calcium current could be observed at all tested membrane potentials, illustrated in Fig. 2Ac, for a 450-ms ramp depolarization from -30 to +50 mV. These experiments strongly suggest that, under our culture conditions, the low resting potential of the neurons is just adequate to maintain a significant proportion of the VGCC calcium channels in the open configuration and therefore enable extracellular calcium to enter into the cytoplasm. This assumption was further verified by monitoring the changes in

the intracellular calcium levels induced by  $K^+$ -induced membrane depolarizations. As illustrated in Fig. 2B, the intracellular calcium level estimated using Fura-2 was found to increase following changes of the extracellular level from 3 to 15 or 30 mM. The F352/F380 ratio increased from  $0.690 \pm 0.002$  in 3 mM  $K^+$  to  $0.713 \pm 0.1$  in 15 mM  $K^+$  and  $0.79 \pm 0.02$  in 30 mM  $K^+$  ( $n = 3$ ). These effects were reversible on return to normal  $K^+$ .

#### Effects of different voltage-gated calcium channel blockers and calcium chelating agents on neuron survival

To assess the role of calcium influx through the VGCC in neuron survival, neurons were incubated in the presence of varying concentrations of non-selective blockers, and the number of surviving neurons was counted 2 or 3 days later and compared with that of untreated (control) neurons of the same age originating from the same culture. We found that the effect of these blockers on neurons survival was different at different stages of the culture.

EFFECTS ON FRESH CULTURES. The cultures were defined as fresh during their first few days after plating ( $\leq 5$  days), the

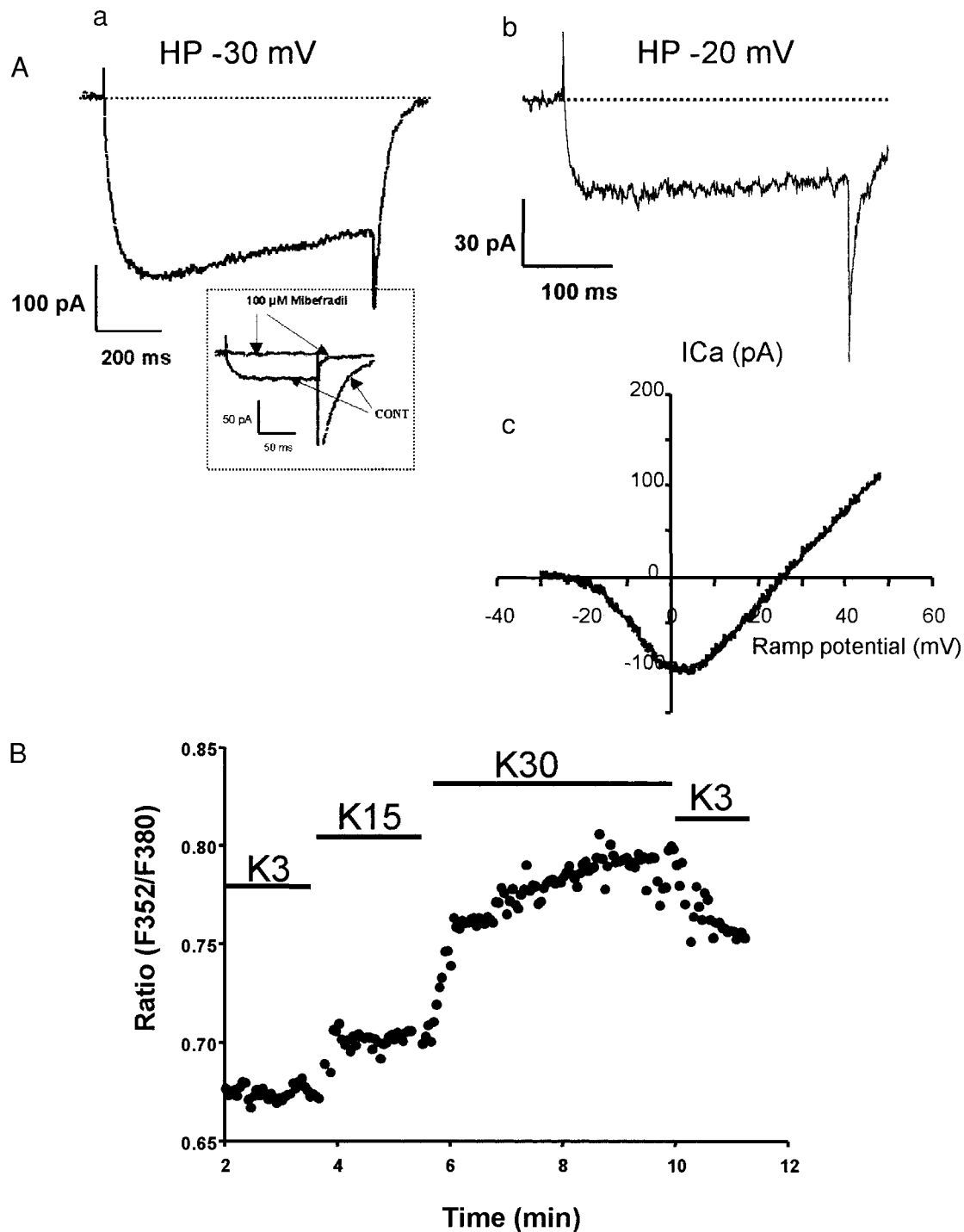


FIG. 2. Calcium currents ( $I_{Ca}$ ) (A) and intracellular calcium level (B) in high potassium media. A: calcium currents recorded from 4 different neurons maintained in the high potassium ( $K = 30$  mM;  $Ca^{2+} = 3$  mM) (L + G) culture medium. The holding potential (HP) was maintained at  $-30$  mV (a, inset, and c) or  $-20$  mV (b) throughout the experiments. a: calcium current induced by a 30-mV square membrane depolarization from a HP of  $-30$  to  $-0$  mV (6 days in vitro neuron). The dotted line indicates 0 current. Inset: inhibition of the current by 100  $\mu$ M mibefradil (same experimental conditions but a different neuron with a much smaller current) b: calcium current induced by a 30-mV square membrane depolarization to 10 mV from a holding of  $-20$  mV. c: current-voltage ( $I-V$ ) relationship for 1 neuron established using a 450-ms ramp depolarization from  $-30$  mV (holding potential) to  $+50$  mV. All neurons from 5- to 6-day-old culture. The leak current was subtracted in all illustrated experiments. B: time course of the changes in the fluorescence ratio (F352/F380, see METHODS) of fura-2-loaded neurons induced by changes in the external potassium concentration (horizontal bars). Upward deflections reflect an increase in the intracellular calcium concentration.

period during which the culture was bathed in the serum-free (5 + 4) medium (see METHODS). During that period, the cell bodies of the neurons had very short neurites, if any. Addition of

mibefradil to the culture medium at concentrations ranging from 0.1 to 100  $\mu$ M led to a dose-dependent neuronal death within 72 h: a significant reduction of the number of neurons

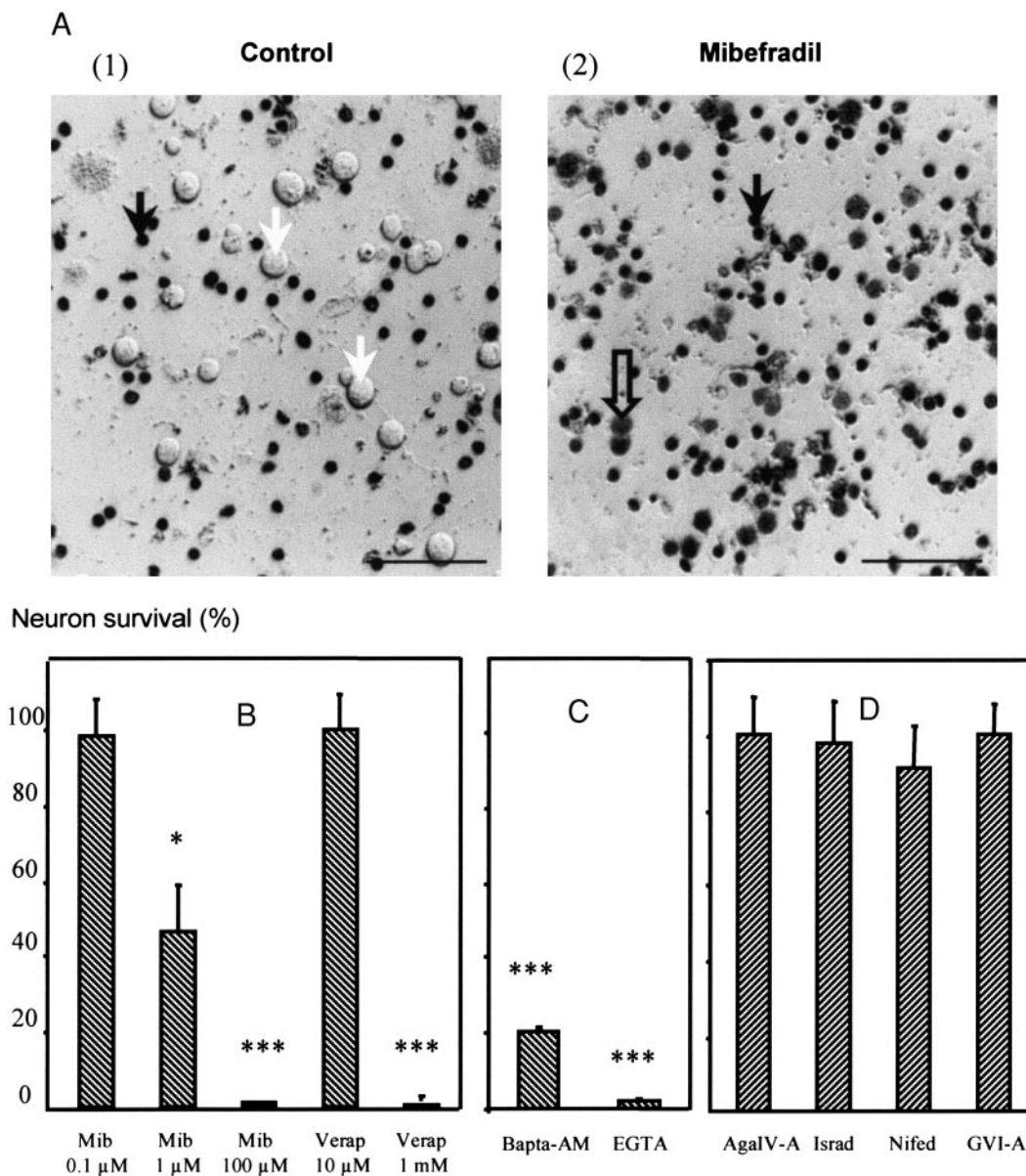


FIG. 3. Effect of voltage-gated calcium channels (VGCC) blockers and calcium chelating agents on survival of freshly cultured neurons in the "5 + 4" culture medium. *A*: effects of a 2-day treatment with 100  $\mu$ M mibefradil on a 3-day-old culture. The blocker was added to the culture medium on day 1. The cultures were observed using Hoffman interference optics 1 h after the addition of trypan blue (20%), which labeled the dead neurons or other cellular debris but not the living cells. Under control conditions (*A1*), 2 categories of round-shaped structures are clearly visible: neurons, which are not labeled by the dye (white arrows), and nuclei of dead cells, which are smaller and strongly labeled (these structures could be fully labeled by nuclear tracers such as Hoechst; data not shown). After treatment with mibefradil (*A2*), the living neurons had disappeared and were replaced by slightly smaller dark structures (black empty arrows) corresponding to the labeled cell bodies of the dead neurons (Note that dead cells have shrunken cell bodies). The scale bars represent 50  $\mu$ m. *B*: effects of 3 concentrations of mibefradil (0.1, 1, and 100  $\mu$ M; Mib) and 2 concentrations of verapamil (0.01 and 1 mM; Verap) on survival. The VGCC blockers were added to the culture media 1 day after plating (1 DIV) and their effects on survival analyzed after 3 days. Six cultures were used for mibefradil and 5 others for verapamil. The results are expressed as percent of control untreated dishes from the same cultures. The average number of neurons per field was  $35 \pm 5$ . \*\*\* $P < 0.001$ . *C*: effects of 10  $\mu$ M BAPTA-AM and 10 mM EGTA on survival. Same protocol as in *B*. Three different cultures were used for each chelator. The average number of neurons per field was  $40 \pm 5$ . *D*: effects of 500 nM  $\omega$ -AgaTxIVA (AgaIV-A), 10  $\mu$ M isradipine (Israd), 10  $\mu$ M nifedipine (Nifed), and 1  $\mu$ M  $\omega$ -CgTx GVIA (GVI-A) on survival. Same protocol as in *B*. The average number of neurons per field was  $30 \pm 5$ . Experiments with  $\omega$ -AgaTx IVA, isradipine, and nifedipine were repeated on 3 different cultures, whereas the experiment with  $\omega$ -CgTx GVIA was done only once. Note that none of the 4 blockers has any significant effect on survival.

was observed at 1  $\mu$ M and all neurons died at 100  $\mu$ M (Fig. 3, *A* and *B*). This lethal effect was correlated with a reduction in the calcium channel current (data not shown). Verapamil had similar effects (Fig. 3*B*) but at higher concentrations (>10

$\mu$ M), as predicted from its lower blocking potency on the current: treatment of 1 day in vitro (DIV) neurons with 100  $\mu$ M and 1 mM verapamil for 3 days resulted in  $50 \pm 20\%$  ( $n = 24$ ,  $P < 0.001$ ) and 100% death of the neurons, respectively.

Addition to the culture medium of 1 mM  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ , or  $\text{Co}^{2+}$  (concentration that completely suppresses  $I_{\text{Ba}}$ ) had the same lethal effect as 100  $\mu\text{M}$  mibefradil and 1 mM verapamil (data not shown). These results suggest that mibefradil and verapamil induced neuronal death in fresh cultures through the inhibition of the calcium influx through the VGCC. However, since relatively high concentrations were needed, it could be argued that the lethal effects were non-specific and therefore not related to a decrease of intracellular calcium. To assess the role of calcium influx in neuronal survival, we prevented the elevation of intracellular calcium using intra- or extracellular calcium chelating agents. Thus about 80% of the neurons loaded with 10  $\mu\text{M}$  BAPTA-AM for 1 h on the first day in culture, washed, and maintained in the control culture media died within 3 days (survival:  $22 \pm 2\%$ ,  $n_f = 52$ ,  $P < 0.001$ ; Fig. 3C). Similarly, reduction of the extracellular free calcium concentration to about 75 nM using 10 mM EGTA (see METHODS) induced the death of more than 90% of the neurons after 3 days (Fig. 3C).

Altogether, the above experiments show that, as in vertebrates, calcium influx through VGCC plays a key role in the survival of embryonic cockroach brain neurons from fresh cultures. The question remained as to which category of VGCC is involved in this survival. We have therefore tested a series of four more specific VGCC blockers: isradipine, nifedipine,  $\omega$ -CgTx GVIA, and  $\omega$ -AgaTx IVA. The results of these experiments are summarized in Fig. 3D. In agreement with their lack of effect on  $I_{\text{Ba}}$  (Benquet et al. 1999), the DHP isradipine (10  $\mu\text{M}$ ) and nifedipine (10  $\mu\text{M}$ ) or the toxin  $\omega$ -CgTx GVIA (1  $\mu\text{M}$ ) had no significant effect on neuron survival. Quite unexpectedly, however, 500 nM  $\omega$ -AgaTx IVA, which was sufficient to suppress about 80% of the current representing the P/Q-like component of  $I_{\text{Ba}}$  (Benquet et al. 1999), had no lethal effect. These results suggest that for these neurons, the R-like VGCC, which are efficiently blocked by mibefradil and verapamil, supports the calcium influx responsible for neuron survival.

EFFECT OF AGING OF THE CULTURE ON THE EFFICACY OF CALCIUM CHANNEL BLOCK IN THE FIRST CULTURE MEDIUM. To determine whether the age of the culture in the first medium could influence the effects of VGCC blockers, neurons were cultured in the "5 + 4" medium without serum for  $\leq 17$  days. As under normal culture conditions (see Fig. 6), the number of living neurons declined slowly with time in culture; the percentages of living neurons relative to the initial number of plated neurons decreased to 84% after 8 days and 82% after 17 days. Under these culture conditions, however, neuritic growth was considerably slowed down and no network was formed. As in the previous experiments with fresh cultures, a 2-day exposure to 100  $\mu\text{M}$  mibefradil was sufficient to kill all neurons at days 2, 7, and 14 (data not shown), indicating that the efficacy of the molecule did not change with time under these conditions.

IS FETAL CALF SERUM ABLE TO PROTECT THE NEURONS AGAINST CALCIUM CHANNEL BLOCKERS? In vertebrate neurons, survival is under the control of neurotrophic factors, some of which are contained in the serum. Serum is also required to obtain a proper in vitro development of embryonic cockroach brain neurons (Beadle and Hicks 1985). It was therefore important to test if, in our preparation, the observed deleterious

effects of VGCC blockers were linked to the lack of serum. To test this hypothesis, we added serum to the first culture medium after 1 day and 2–4 days later tested the effects of 100  $\mu\text{M}$  mibefradil. After 2–3 days, all tested neurons were dead. This experiment was repeated on three different cultures and gave the same negative result. To check if, in these fresh cultures, the lethal effects were not due to the nature of the culture medium, the same experiment was repeated with the second medium (+serum), which was substituted for the first medium after the first day in culture. The results were essentially the same.

EVOLUTION OF THE EFFECT OF MIBEFRADIL DURING THE FORMATION OF THE NETWORK. For this part of the study, the culture protocol was modified; the neurons were switched to the second culture medium plus 10% calf serum after only 1 day in the first medium to boost their development. Under those conditions, neurons started migrating within 2 days to form small aggregates of two to six somata and extended neurites, while the average number of neurons per field fluctuated from 25 to 34 in a non-significant manner (Fig. 4). The number of neurons bearing neurites increased progressively with time in culture, leading to the establishment of a neural network after 1–2 wk. At the same time, the number of isolated neurons dropped from about 38% a few hours after switching to the serum containing medium to  $< 5\%$  at day 4 (see also Angevin et al. 2000). After about 1 wk, 50% of the neuronal population consisted of small clusters of neurons that were not yet connected to the network. Under these experimental conditions, when mibefradil (100  $\mu\text{M}$ ) was added to the "L + G" serum-containing medium for 2 days after 0, 2, 4, or 6 days in culture, a significant proportion of neurons died, as expected from the previous experiments. Interestingly, however, the number of residual neurons which survived after the 2-day treatment with the blocker increased with time in culture. Thus in the experiment illustrated in Fig. 4, the proportion of surviving neurons rose from 0% on day 2 ( $n_f = 12$ ) to  $5 \pm 1\%$  ( $n_f = 14$ ) at day 4,  $36 \pm 6\%$  ( $n_f = 14$ ) at day 6, and  $58 \pm 12\%$  ( $n_f = 15$ ) at day 8. This increase paralleled the development of the neurons in the control dishes as expressed by the proportion of neurons bearing neurites. The same test was repeated three times on three different cultures with similar results, showing that, under these experimental conditions, a resistance to mibefradil-induced neuronal death appeared progressively with the same course as neurite acquisition. This result might indicate that the presence of neurites, which enable cell-cell interaction during network formation, is a key factor that rescues neurons from death.

EFFECTS ON CONNECTED NEURONS IN MATURE CULTURES. Cultures were considered mature when most neurons had established strong connections with the neighboring neurons to form a complex network consisting of ganglion-like clusters of neurons connected to each other by straight connectives corresponding to the coalescence of individual neurites (see Figs. 5A, 6A, and 7A). Under our standard culture conditions, despite small variations from culture to culture, this state was reached in the second medium after 10 DIV. When this network was established, the diameter of the connectives continued to increase to reach a maximum after 6–8 wk. As suggested in Fig. 5B, this increase resulted from the combined effects of neurite outgrowth, a process enabling the connectives to recruit neu-



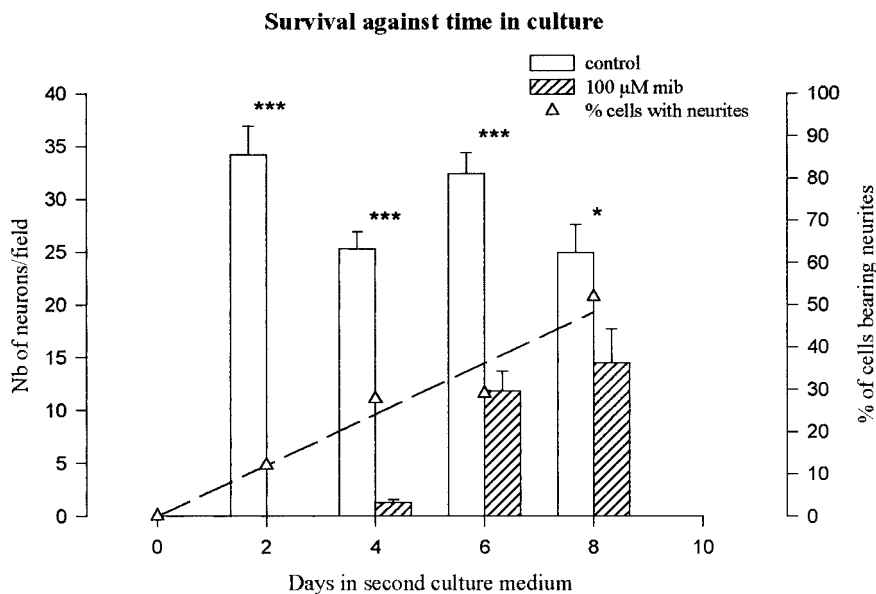


FIG. 4. Typical example of the evolution with time in culture of the resistance of the neurons to a 2-day exposure to 100  $\mu\text{M}$  mibefradil (bars, left scale) and its relationship with neurite outgrowth ( $\Delta$ , right scale). After 1 day in the 1st medium (5 + 4), the culture was switched to the 2nd medium (L + G) + 10% fetal calf serum, which enabled neurite outgrowth. Every 2 days, 100  $\mu\text{M}$  mibefradil was added to 2 culture dishes, and the number of living neurons were counted 2 days later in this treated dishes and in 2 control dishes from which the proportion of neurons bearing neurites was also computed. Whereas the number of living neurons slightly decreased with time (open bars), the number of neurons surviving the treatment (hatched bars) increased in parallel with the percentage of cells bearing neurites when the VGCC blocker was added, suggestive of an inverse correlation between the development of neurites and the toxicity of the molecule. Vertical bars:  $\pm$ SE. The linear regression through the points representing neuritic outgrowth (dotted line) yielded a slope of 6 and regression coefficient of 0.95. Each data point corresponded to  $\geq 14$  fields. The abscissa correspond to the actual number of days in culture -1 (the first day during which the neurons were kept in the 1st culture medium). \*\*\*level of significance of the difference between treated and non-treated cultures computed using the unpaired Student's *t*-test as indicated in METHODS.

rites from more and more distant neurons and branching from neurons that already contributed to the nerve trunk (see Fig. 5A, 3 and 4) (see also Beadle and Hicks 1985 and Angevin et al. 2000). In the experiment illustrated in Fig. 5, the mean length of the longest neurites increased from  $77.1 \pm 15.4$  ( $n = 7$ ) for neurons from 5 to 10 DIV cultures to  $193.3 \pm 29$  ( $n = 6$ ;  $P = 0.004$ ) for neurons from 20–25 DIV cultures; for these same neurons and during the same period, the mean number of ramifications increased from  $8.6 \pm 2.4$  ( $n = 7$ ) to  $15.5 \pm 2.8$  ( $n = 6$ ;  $P = 0.09$ ).

Preventing calcium influx through VGCC with a saturating concentration (100  $\mu\text{M}$ ) of mibefradil failed to affect survival in these cultures as illustrated in Fig. 6. This same lack of effect has been observed on the five tested cultures. A similar result was obtained on 12 to 25 DIV cultures following a 2-day or more pretreatment with verapamil (1 mM),  $\omega$ -AgaTx IVA (500 nM), 10 mM EGTA, or a 1-h load with 50  $\mu\text{M}$  BAPTA-AM; the percentages of surviving neurons following these pretreatments were  $98 \pm 7\%$  ( $nf = 15$ ,  $P = 0.8$ ),  $107 \pm 7\%$  ( $nf = 32$ ,  $P = 0.4$ ),  $106 \pm 7\%$  ( $nf = 15$ ,  $P = 0.5$ ), and  $107 \pm 4\%$  ( $nf = 52$ ,  $P = 0.3$ ), respectively. The lack of effect of organic VGCC blockers and calcium chelating agents on survival of neurons from mature cultures indicates that the properties of the neurons change during *in vitro* development. As suggested by the experiments illustrated in Fig. 4, this modification is associated with the formation of the network.

#### Effect of voltage-gated calcium channel blockers and BAPTA-AM on neurite outgrowth

EFFECTS ON THE DIAMETER OF THE LARGEST CONNECTIVES. Addition (for  $\geq 2$  days) of concentrations of mibefradil that totally suppressed  $I_{\text{Ba}}$  (100  $\mu\text{M}$ ) resulted in a clear inhibition of the increase in diameter of the largest connectives compared with that of control untreated neurons of the same age and same culture (Fig. 6, A and C). With 10  $\mu\text{M}$  mibefradil, the effect was already significant after 5 days of incubation and could be maintained for periods lasting at least 2 wk and was partly reversible on withdrawal of the blocker (Fig. 6C). One simple

explanation might have been that the decrease reflected a reduction of the number of surviving neurons (cf. Fig. 3B). This interpretation can be ruled out since, under the same experimental conditions, there is no detectable difference in cell density between treated and control cultures (Fig. 6, A and B). This has been found to be true for at least five different cultures. The same phenomenon was also seen with higher concentrations of verapamil: a 4-day pretreatment with 10 and 100  $\mu\text{M}$  verapamil yielded mean largest connective diameters of  $92 \pm 9\%$  ( $nf = 38$ ,  $P > 0.05$ ) and  $55 \pm 6\%$  ( $nf = 34$ ,  $P < 0.05$ ), respectively, of the control values. Interestingly, for three different cultures, 500 nM of the P/Q-type calcium channel blocker,  $\omega$ -AgaTx IVA (which had no effect on neuronal survival, even at saturating concentration) reduced the diameter of the connectives as illustrated in Fig. 7. The effect of the toxin on the diameter of the largest connective was significant after 4 days of incubation and could be maintained for at least 1 wk. Despite the observation that the effect of the toxin on the diameter of connectives was often less pronounced than that of 100  $\mu\text{M}$  mibefradil for the same incubation period (suggested from the larger *P* values obtained from the Student's *t*-tests), this result shows that the P/Q-like VGCCs are involved in neurite outgrowth. As expected, a significant reduction of the diameter of the largest connective ( $25 \pm 4\%$ ,  $nf = 36$ ,  $P < 0.05$ ) was also found 72 h after a 1-h pretreatment of a 13 DIV culture with 50  $\mu\text{M}$  BAPTA-AM.

EFFECTS OF MIBEFRADIL ON NEURITE DIAMETER. As mentioned earlier, the diameter of the connectives reflects neurite outgrowth and their ramifications. In a second series of experiments, we analyzed the structural modifications underlying the changes in diameter of the connectives in the presence of 100  $\mu\text{M}$  mibefradil. Scanning electron micrographs of typical connectives of 14-day-old control cultures were compared with those of similar cultures after a 4-day pretreatment with 100  $\mu\text{M}$  mibefradil (Fig. 8A); the control connectives were clearly thicker and made of a bunch of neurites, whereas the treated connectives were much thinner and contained only a few neurites. Importantly, however, the mean diameter of individual neurites was not significantly different between control ( $0.21 \pm 0.02$   $\mu\text{m}$ ,  $n = 31$ ) and



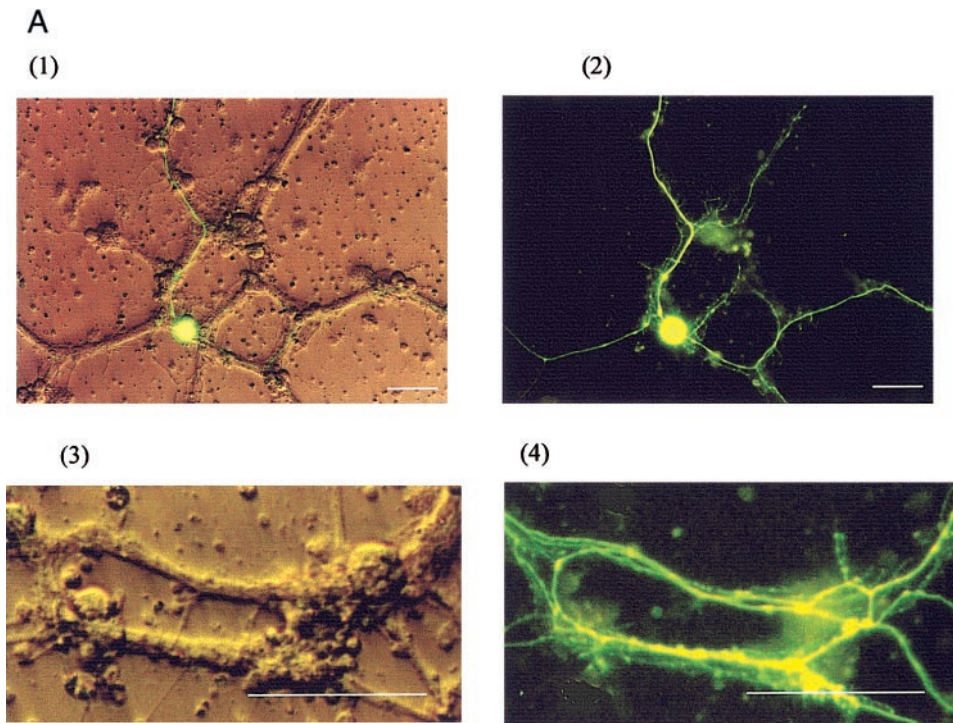
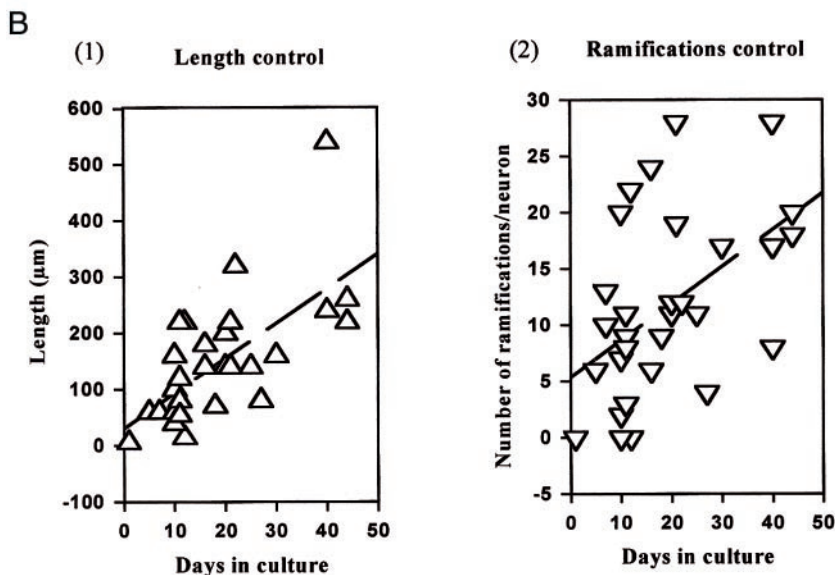


FIG. 5. A: correspondence between the gross morphology of the network and the morphology of individual neurons as visualized following injection of Lucifer yellow into the cell body. In *panels 1 and 3*, the cultures were viewed using a combination of epifluorescence illumination and Hoffman interference. In *panels 2 and 4*, the same cultures were viewed under epifluorescence illumination only, enabling a much better resolution of the morphology of the injected neurons. The culture was 43 days old in *panels 1 and 2* and 44 days old in *panels 3 and 4*. The scale bars correspond to 50  $\mu\text{m}$ . In *panels 1 and 2*, it is clear that the same neuron sends neurites in several directions and therefore participates to several connectives of the network. In *panels 3 and 4*, which were taken at a higher magnification, the complex geometry of the labeled neuron is clearly illustrated; the same neuron exhibits numerous ramifications, some of which run in parallel in the same connective. B: development of individual neurons in culture as estimated using Lucifer yellow: (1) Time-dependent increase in the length of the longest neurite ( $\Delta$ ) for 31 injected neurons. (2) Time-dependent increase of number of 1st-3rd order ramifications ( $\nabla$ ) for the same 31 neurons. Both parameters increased with time in culture [dashed line with slopes of, respectively, 6.2 (length) and 0.32 (ramifications) and  $r^2$  values of 0.47 and 0.24].



treated cultures ( $0.26 \pm 0.04 \mu\text{m}$ ,  $n = 18$ ,  $P = 0.26$ ; Fig. 8C). These results demonstrate that, under our experimental conditions, the reduction of the nerve trunk diameter reflects a reduction in the number of neurites per connective and not a reduction of the diameter of individual neurites.

**EFFECTS OF MIBEFRADIL ON THE MORPHOLOGY OF INDIVIDUAL NEURONS.** The next step was to characterize the effects of the channel blockers on the morphology of individual neurons. To do so, we perfused 44 neurons with Lucifer yellow and counted, for each neuron, the number of primary neurites and the number of first to third order ramifications and measured the length of the longest neurite: 31 neurons came from untreated (control) culture and 13 from cultures that had been exposed for 6 days to 100  $\mu\text{M}$  mibefradil. For 33 neurons, which had been in culture for 10–25 days, the number of

primary neurites was  $1.62 \pm 0.33$  versus  $2.95 \pm 0.35$  in control conditions ( $P = 0.014$ ), the number of ramifications was  $3.15 \pm 0.85$  versus  $11.75 \pm 1.74$  in control conditions ( $P = 0.0007$ ), and the mean length of the longest neurite was  $93.8 \pm 22.8$  versus  $149.2 \pm 18.8 \mu\text{m}$  in control conditions ( $P = 0.07$ ; Fig. 8, B and C). This result suggests that calcium influx is important in several processes that are implied in the morphogenesis of individual neurons, and it would be interesting to dissect out which calcium current components are involved in the branching mechanism or the lengthening of the neurites.

#### DISCUSSION

The goal of this study was to determine the contribution of VGCC to the *in vitro* development of embryonic cockroach brain neurons.

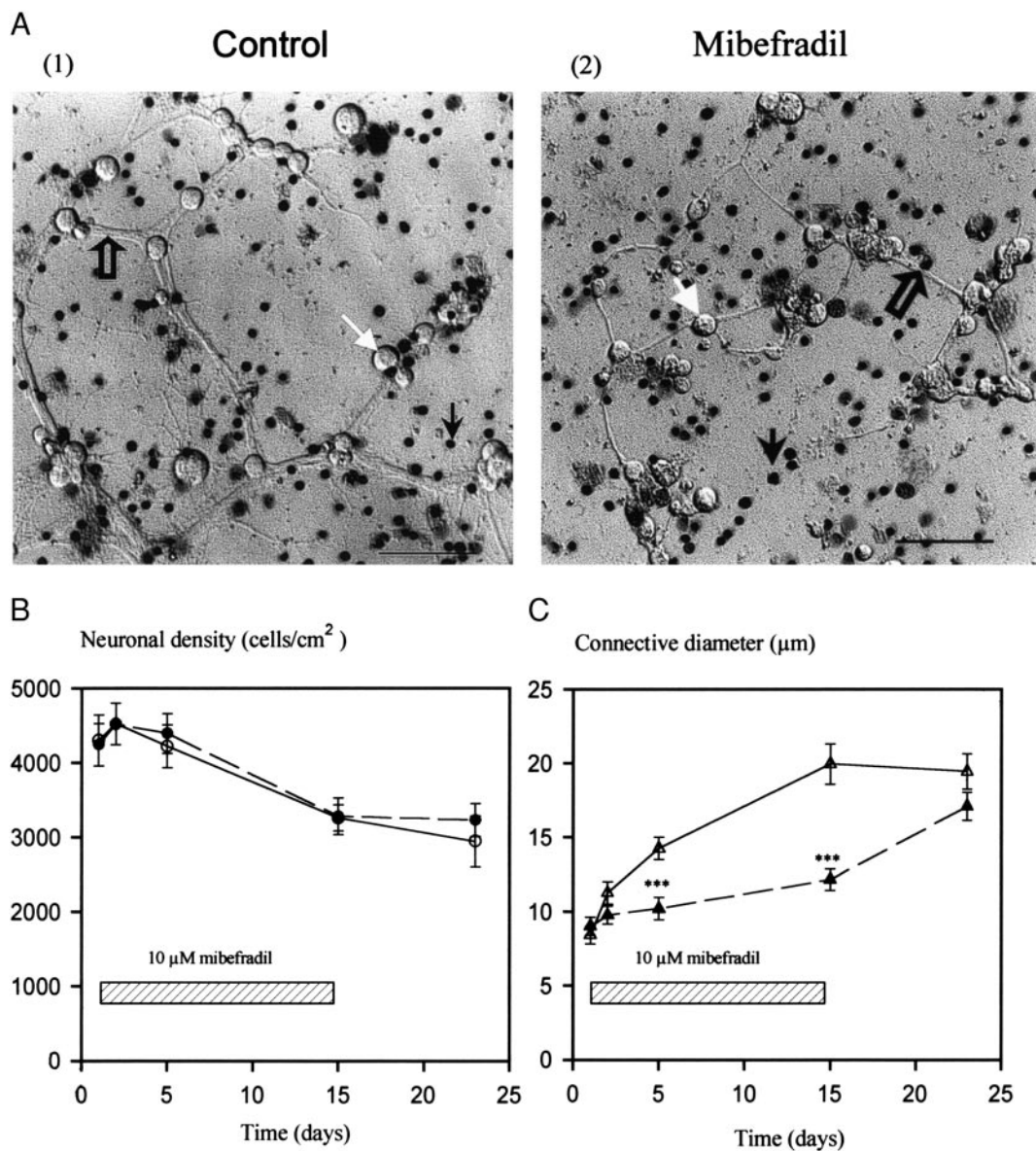


FIG. 6. Effect of mibefradil on neuronal survival and connective diameter in developing cultures in the L + G serum-containing medium. A: digitized pictures of 17 DIV neurons cultured in the absence (1) or presence of 100  $\mu\text{M}$  mibefradil (2). VGCC blocker was added in the culture medium at 11 DIV. The pictures were obtained in the same conditions as in Fig. 3A. White arrows indicate living neurons and black arrows indicate nuclei of dead cells. Black empty arrows indicate connectives between groups of neurons. Note that, in both cases, a network was formed and the neurons were alive but the diameter of the connectives was much smaller in treated cultures than in control conditions. Scale bars correspond to 50  $\mu\text{m}$ . B: graph illustrating the time-dependent evolution of the neuronal density in the absence (○) and presence of 10  $\mu\text{M}$  Mibefradil (●) during the period indicated by the hatched bar. Vertical bars correspond to  $\pm$ SE. Values were taken from 5 different cultures. Mibefradil had no detectable lethal effect. C: graph representing the time-dependent evolution of the average diameter of the largest connectives per 0.5-mm<sup>2</sup> fields in the absence (Δ) and presence of 10  $\mu\text{M}$  mibefradil (▲) during the period indicated by the hatched bar. The difference between control values and mibefradil-treated cultures was already significant after 5 days of incubation and even more so after 15 days (\*\*\* $P < 0.001$ , unpaired Student's *t*-test). This effect was partially reversible following return to drug-free medium. Results illustrated in B and C were obtained from the same batch of 11–15 DIV cultures. Each data point corresponded to  $\geq 30$  fields.

The main findings (Fig. 9) can be summarized as follows. Agents that alter directly calcium influx through VGCC and/or the intracellular calcium concentration affect survival and growth of cultured cockroach neurons. The effects are different for “immature” neurons that are not part of a network and for “mature” neurons that are connected to other neurons, forming a more or less extensive neuronal network: the non-selective VGCC blockers mibefradil and verapamil, as well as EGTA

and BAPTA-AM, induce neuronal death for the former but have no lethal effect on the latter. Surprisingly, the P/Q-type calcium channel blocker,  $\omega$ -AgaTx IVA, has no lethal effect on “immature neurons.” None of the blockers were found to have any significant effect on survival of “mature” neurons, and this resistance appeared to be related to the existence of neurites and not to the presence of serum in the culture medium or to the age of the culture. VGCC blockers (in-



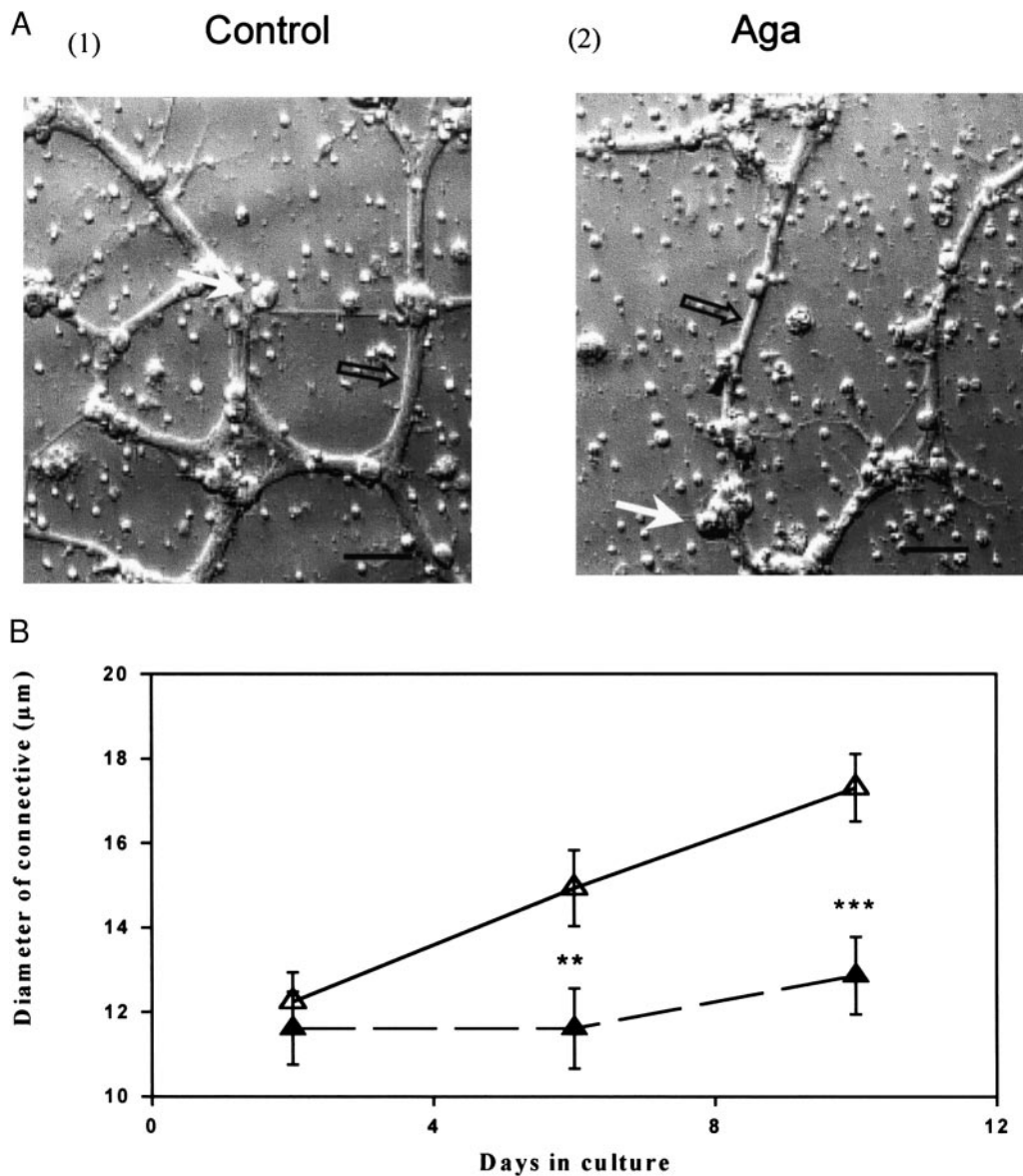


FIG. 7. Effects of 500 nM  $\omega$ -AgaTx IVA on the diameter of the connectives in developing neuronal cultures in the L + G serum-containing medium. **A**: digitized pictures of 16 DIV neurons cultured under normal conditions (Control) or after the addition in the culture medium, 5 days before, of 500 nM  $\omega$ -AgaTx IVA (Aga). Trypan blue was not used in these experiments, but living neurons (white arrows) could still be clearly distinguished from the cellular debris. As in Fig. 6, the diameter of the connectives (black open arrows) were larger in control conditions than in treated neurons. Scale bars correspond to 50  $\mu\text{m}$ . **B**: graph illustrating the time-dependent evolution of the average diameter of the largest connectives (in  $\mu\text{m}$ ) under control conditions ( $\Delta$ ) and in the presence of 500 nM  $\omega$ -AgaTx IVA ( $\blacktriangle$ ). This result was obtained with 3 different cultures.  $\omega$ -AgaTx IVA inhibits the increase in diameter, the effect being already highly significant after 5–6 days (23.5  $\pm$  3% reduction,  $n_f = 74$ ,  $**P < 0.01$ , unpaired Student's  $t$ -test). The vertical bars correspond to  $\pm$ SE.

cluding  $\omega$ -AgaTxIVA), as well as BAPTA-AM, were found to inhibit the increase in diameter of the connectives with time in culture. The effects of mibefradil on the morphology of the cultured neurons indicate that, at least for that molecule, the difference in diameter between treated and control cultures is not related to a change in the diameter of individual neurites, visualized with a scanning electron microscope, but to a significant decrease in the number of primary and secondary branchings and a detectable (but not significant) decrease in neuritic length.

These results, together with the demonstration that VGCC enable a sizeable calcium current to flow at rest under our standard

culture conditions, illustrate that, as in many vertebrate neuronal culture systems, calcium plays a prominent role in survival and development of nerve cells. They also indicate that R-like channels and P/Q-like channels play a differential role.

#### *Mibefradil is the most efficient blocker of the VGCC of embryonic cockroach neurons*

The effects of mibefradil on the currents flowing through the calcium channels are in good agreement with those on VGCC of freshly isolated, ex vivo, developing neurons of the same preparation (Benquet et al. 2000) and those observed on in



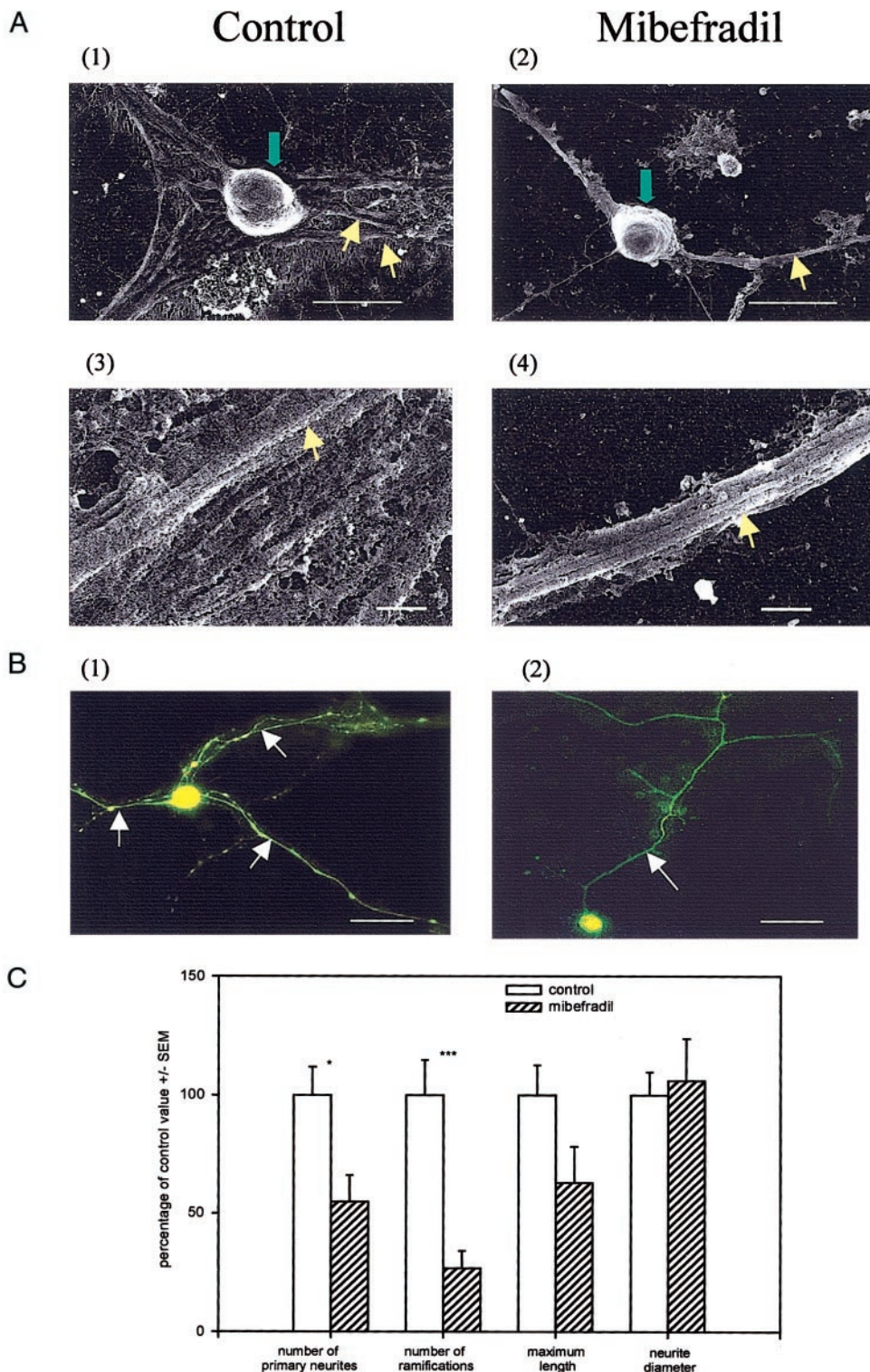


FIG. 8. Effects of 100  $\mu$ M mibefradil on the morphology of the cultured neurons. *A*: scanning electron micrographs of typical connectives found on 14-day-old cultures under control conditions (1 and 3) or after 4 days pretreatment of the neurons with 100  $\mu$ M mibefradil (2 and 4). Thick green arrows indicate the cell body and the thin yellow arrows indicate individual neurites. Note that the scale is different in (1 and 2) and (3 and 4): the scale bar corresponds to 10  $\mu$ m in the former 2 panels and to 1  $\mu$ m in the latter panels, reflecting the 5 times increase in magnification that was necessary to distinguish individuals neurites in the connectives. Pretreatment with mibefradil had no effect on the diameter of individual neurites but reduced considerably their number and thereby the overall diameter of the connectives compared with controls. *B*: digitized pictures illustrating the typical morphology of Lucifer yellow-perfused neurons from control (1) or after a 6-day pretreatment of neurons with 100  $\mu$ M mibefradil (2). Both neurons were from the same 14 DIV culture. Note that the number of primary neurites (white arrows) is reduced in the presence of mibefradil. Scale bars correspond to 30  $\mu$ m. *C*: histograms summarizing the effects of a 4-day pretreatment with 100  $\mu$ M mibefradil on the number of primary neurites, the number of ramifications, and the length of the longest neurite for 20 control neurons and 13 treated neurons from 10- to 25-day-old cultures. The mean diameters of the neurites were estimated from scanning electron micrographs of 31 neurites from control cultures and 13 neurites from treated cultures. The results are expressed in percent of control values  $\pm$  SE (or SD for the diameter of neurites). \*\*\*level of significance of the differences between treated and non-treated cultures computed using the unpaired Student's *t*-test. \**P* = 0.016; \*\**P* = 0.0007.

vitro vertebrate neuronal preparations such as embryonic rat spinal motoneurons (Viana et al. 1997), NG108-15-derived neurons (Randall and Tsien 1997), freshly isolated rat cerebellar Purkinje neurons (McDonough and Bean 1998), and freshly dissociated dorsal root ganglion neurons of adult rats (Todorovic and Lingle 1998). Mibefradil was found, however, to be less potent in other preparations or on other HVA calcium currents (McDonough and Bean 1998; Niespodziany et al.

1999). Furthermore, in a few cases, and so far, on non-nervous preparations, the molecule has been found to block other channels than the VGCC (Chouabe et al. 1998; Hahn et al. 1995; Liu et al. 1999; Nilius et al. 1997). Therefore, one cannot exclude that, in our experiments, mibefradil had a non-specific effect, but this effect, if present, was probably minimal compared with verapamil for which non-specific effects on potassium currents have been described for several preparations

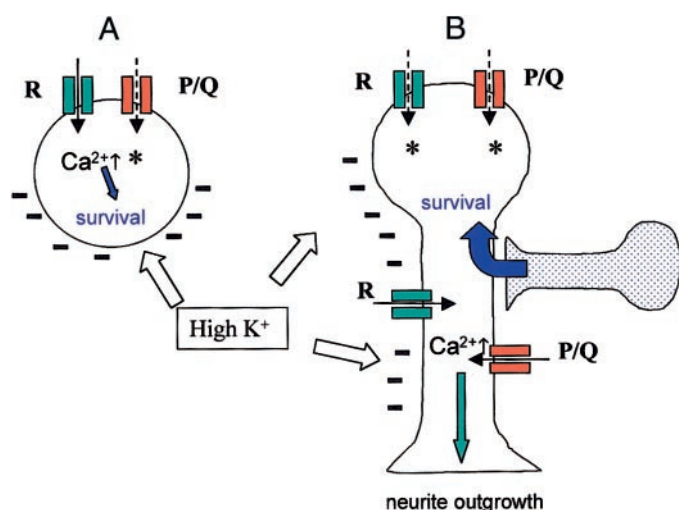


FIG. 9. Schematic diagram illustrating the hypothetical role of the VGCC on survival and neuritic outgrowth of "immature" (A) and "mature" (B) cultured neurons. In both cases, the neurons are bathed in a high potassium culture medium that depolarizes the membrane (as shown by the minus sign) and opens the 2 categories of high-voltage activated calcium channels:  $\omega$ -AgaIVA-sensitive (P/Q) channels (red pores) and  $\omega$ -AgaIVA-resistant (R) channels (green pores), which are present in the neuronal membrane. In fresh cultures (A), survival (green arrow) is associated with the R-like channels and not the P/Q-like channels (dotted arrow). In older cultures (B), none of the calcium channel blockers has any direct effect on survival. It is suggested that, at that stage of development, survival is due to an unknown interaction (green curved arrow) between the connected neurons. The switch from A to B occurs in parallel with neurite outgrowth (green arrow), which is itself under the dependency of an entry of calcium through the 2 categories of channels that are shown here located on the elongated part of the neuron.

(Chouabe et al. 1998; Trequattrini et al. 1996, 1998). For the present analysis, we needed to probe the effects of the different calcium current components, and therefore we could not restrict ourselves to the use of the toxin  $\omega$ -AgaTx IVA, which is a potent blocker of the P/Q-like component of VGCC currents of these neurons but does not block the R-component of the current (Benquet et al. 1999). This is the reason why we used mibefradil; it was preferred to verapamil because it was more potent and likely to be more specific.

#### *K<sup>+</sup>-enriched culture media induce calcium influx through VGCC and survival of "immature" neurons*

Our observation that the calcium conductance can be activated at low resting potentials, in our culture conditions, is in agreement with our previous experiments (Benquet et al. 1999) in which we had shown that one-half activation of the calcium conductance was obtained for  $-10$  mV and one-half inactivation for  $-30$  mV. The values of the resting potential measured in the present experiments (around  $-30$  mV in the first medium and around  $-20$  mV in the second medium for 16 and 30 mM potassium, respectively) are in general agreement with those predicted from the curve given by Lees et al. (1985) for these same neurons in primary culture. Interestingly, this range of membrane potentials has been found by Franklin et al. (1995) to be optimal in preventing programmed neuronal death in rat sympathetic neurons in vitro (90–100% survival at about  $-21$  mV). The effects of high-potassium solutions on survival of cultured neurons have been described in several preparations, including dissociated dorsal root ganglia (Collins and

Lile 1989; Scott 1979), rat cerebellar granule cells (Gallo et al. 1987), chick embryonic ciliary neurons (Collins and Lile 1989; Collins et al. 1991), and sympathetic neurons (Collins and Lile 1989; Koike et al. 1989). In general, neurons survival, in high external potassium-induced membrane depolarization, was associated with an entry of calcium into the cell through VGCC (Becherer et al. 1997; Collins et al. 1991; Franklin et al. 1995; Scamps et al. 1998; Toescu 1999). Conflicting results concerning changes in cytosolic calcium in high- $K^+$  solutions was reported on cultured rat sympathetic neurons (Franklin et al. 1995; Murrell and Tolkowsky 1993) and quite unexpectedly, Ono et al. (1997) and Kohara et al. (1998), working on cultured rat cerebellar granule cells, found no significant changes in cytosolic calcium in high- $K^+$  solutions and suggested that survival might be due to an increase in the turnover of calcium. In the present experiments we have indeed shown that increases of external  $K^+$  from 3 to 15 or 30 mM induced a clear and reversible increase in intracellular calcium, an increase that could be inhibited by 50  $\mu$ M mibefradil.

In vertebrates, neuron survival is generally associated with the activation of HVA L-type VGCC (Blair et al. 1999; Collins and Lile 1989; Collins et al. 1991; Gallo et al. 1987; Koike et al. 1989; Murrell and Tolkowsky 1993; Yano et al. 1998). This DHP-sensitive calcium channel was not found in cultured embryonic cockroach brain (Benquet et al. 1999, 2000). Our observation that the toxin,  $\omega$ -AgaTx-IVA, which blocks the major component (P/Q-type) of the calcium current, has no effect on survival, is not artifactual, and is not due to the degradation of the toxin molecule during the 3-day incubation period, since this same toxin was still active on axonal outgrowth after much longer incubation periods (1 wk, see Fig. 7). This result therefore suggests that the R-like but not the P/Q-like VGCC are involved and sufficient to support neuron survival. A direct demonstration of the implication of this type of channel awaits specific blockers.

The mechanism(s) by which calcium promotes cell survival is yet unknown. During the past few years, evidence has accumulated that the route by which calcium enters the cytosol is important in determining which intracellular signaling pathway(s) is recruited, and subsequently, which genes are expressed (Bading et al. 1993; Brosenitsch and Katz 2001; Hardingham et al. 1997, 1999; Hu et al. 1999; Lerea and McNamara 1993). The probable existence of different pathways between the  $\omega$ -AgaTxIVA-sensitive P/Q-like channels and  $\omega$ -AgaTxIVA-resistant R-like channels might account for the differential effects of the corresponding blockers on survival and neurite outgrowth of embryonic cockroach neurons in the high  $K^+$  culture media.

#### *Survival of "mature" network-connected neurons is independent of calcium entry through VGCC*

The lack of effect of VGCC blockers and calcium chelating agent on survival of neurons involved in network formation suggest a switch from calcium-dependent to a calcium-independent survival mechanism between "immature" and "mature" cultured neurons. This switch could arise from several different mechanisms. It could correspond to a developmental uncoupling of membrane depolarization and nuclear calcium elevation (Birch et al. 1992; Holliday et al. 1991; Kocsis et al. 1994a,b) and be triggered by the development of cell-cell



contacts when neurons are connected to each other in the network (for review see Doherty and Walsh 1991, 1994, 1996). It could also be linked to the release of neurotransmitters (Nguyen et al. 2001; van den Pol et al. 1992) more or less independently of extra- or intracellular calcium or be mediated by diffusible autocrine or paracrine neurotrophic factor(s) as recently hypothesized for invertebrates neurons (Lucini et al. 1999; van Kesteren et al. 1998). It is worth mentioning in this respect that, when the network is formed, neurites exhibit numerous varicosities that have been shown to contain clusters of clear and dense core vesicles (Beadle and Hicks 1985; Beadle et al. 1982). More experiments are needed to clarify this point.

#### Calcium influx through VGCC-enhanced neurite outgrowth

The inhibitory effects of calcium channel blockers on neurite outgrowth has also been found in other preparations such as trypsin-dissociated neurons from chick retinal and muscle cocultures (Suarez Isla et al. 1984) or cultured buccal ganglion neurons from the mollusc *Helisoma trivolvis* (Mattson and Kater 1987). Calcium channel block and/or calcium depletion do not always reduce neuritic growth, and conversely, increased intracellular calcium levels do not necessarily induce neuritic outgrowth. Thus, in neuroblastoma NIE-115 cells (Silver et al. 1989), *Xenopus* spinal cord neurons in vivo (Gomez and Spitzer 1999), and in cultured nerve cord explants from the crayfish *Procambarus clarkii* (Lnenicka et al. 1998) elevated cytosolic calcium inhibits either growth cone elongation or axonal regeneration. The difference between these two sets of conflicting observations concerning the effects of calcium on neuritic outgrowth could result from several different factors: difference in cell specificity, differences in the developmental stage, differences in the nature, and the density of the calcium channels.

Our observation that (non-L-type) HVA calcium channels are involved in the regulation of neurite outgrowth fits in with previous findings in both vertebrates and invertebrates neurons. Thus in cultured crayfish axons, the activity-dependent inhibition of axon regeneration would be under the control of a P-type calcium channel (Hong and Lnenicka 1997). Similarly, according to Heng et al. (1999), cultured rat retinal ganglion cells morphology would be shaped by the P- and Q-type VGCC that modulate the number and length of neurites without affecting their initiation.

#### Physiological relevance of the effects of high- $K^+$ concentrations

An important point that needs discussion is the physiological relevance of our in vitro model in "high- $K^+$ " culture media. In other words, is it possible that, in vivo, the fluid that surrounds the differentiating neurons contains potassium levels equivalent to those used in the present experiments. In the absence of reliable data on the ionic environment of individual neurons in vivo, it is not possible to give a definite answer to that question. However, there are reasons to believe that, in the case of our insect model, *Periplaneta americana*, it may be the case. In a detailed study of the ionic content of the hemolymph of this insect, it has been shown (Pichon 1970) that the potassium concentration was very high (around 60 mM) in oothecae and

young larvae. Furthermore, there is indirect evidence that the "extracellular" fluid that surrounds the nerve membrane also contains high potassium levels (Pichon and Boistel 1968).

The results presented in this paper strongly suggest that, as in vertebrates, calcium influx through VGCC plays an important role in survival and neurite outgrowth of insect embryonic neurons in vitro. These results are also original in several respects. To our knowledge, this is the first report showing that 1) as in vertebrates, mibefradil potently and efficiently reduced VGCC of an invertebrate neuron, and 2) two different non-L-type HVA VGCC are differentially involved in neuron survival and neurite outgrowth. Furthermore, our results indicate that the early expression of different types of VGCC during neuronal development might be an upstream mechanism allowing the discrimination of different cellular functions triggered by the (otherwise) ubiquitous intracellular calcium ions. Finally, our observations on an insect model strongly suggest that the basic mechanisms of neuronal survival and neurite outgrowth have been conserved during phylogeny.

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