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POST-NATAL DISAPPEARANCE OF TRANSIENT CALCIUM CHANNELS IN MOUSE SKELETAL MUSCLE: EFFECTS OF DENERVATION AND CULTURE

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SUMMARY

1. The whole-cell voltage clamp technique was used to record Ba^{2+} currents in voltage-sensitive Ca^{2+} channels in mouse flexor digitorum brevis muscles developing *in situ* from day 1 to 30 after birth. Effects of denervation and tissue culture on the Ca^{2+} channel currents were also studied.

2. The muscle fibres in newborn mice showed two distinct types of Ca^{2+} channel currents, a low-threshold transient current and a high-threshold sustained current.

3. The specific amplitude of the transient current was $2{\cdot}7 \pm 1{\cdot}7$ (S.D.) A/F in response to -30 mV test pulses in medium containing 30 mM-Ba^{2+} on day 1 after birth. The transient current decreased progressively in the post-natal days and became undetectable by day 17. In contrast, the specific amplitude of the sustained current in response to $+20$ mV test pulses increased 4-fold from 6·9 A/F on day 1 to 27·7 A/F on day 30.

4. The disappearance of the transient current could not be accounted for by either shifts in voltage dependence of activation and inactivation or changes in activation and inactivation times of the two types of current during development.

5. Denervating muscle fibres on day 8 after birth did not prevent the disappearance of the transient current. Denervating them on day 17 did not allow reappearance of the transient current. However, the increase of the sustained current was suppressed by the denervation either on day 8 or day 17.

6. In muscle fibres isolated on day 8 after birth and cultured thereafter, the transient current did not disappear until day 19 in culture (27 days after birth), while the sustained current was maintained at the level on day 8.

7. In muscle fibres isolated on day 17, when the transient current had become undetectable, and cultured therafter, the transient current did not reappear until day 15 in culture (32 days after birth), while the sustained current was maintained at a level similar to that on day 17.

8. We conclude that innervation has little influence on the developmental disappearance of the transient Ca^{2+} channel current in mouse muscle fibres, and suggest that some influencing factors from surroundings other than the nerve may be required for the disappearance of the functional transient channels.

INTRODUCTION

Calcium channels are phylogenetically one of the oldest ion channels, and they are found in a wide variety of cell types (e.g. Hagiwara, 1983; Hille, 1984). There are several kinds of Ca^{2+} channels which differ in voltage sensitivity, opening kinetics and chemosensitivity. Although the voltage-sensitive Ca^{2+} channels in some cells appear to be of a single type, many other cells have more than two distinct types of voltage-sensitive Ca^{2+} channels as revealed by the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Most cells contain transient type and sustained type Ca^{2+} channels, known as the T-type and L-type in chick dorsal root ganglion neurones (Nowycky, Fox & Tsien, 1985) and guinea-pig ventricular cells (Nilius, Hess, Lansman & Tsien, 1985), 'transient' and 'sustained' or 'slowly inactivating' in vascular smooth muscle cells and a smooth muscle cell line (Sturek & Hermsmeyer, 1986; Loirand, Pacaud, Mironneau & Mironneau, 1986; Friedman, Suarez-Kurtz, Kaczorowski, Katz & Reuben, 1986) and by a variety of other classifications in different preparations (Fedulova, Kostyuk & Veselovsky, 1985; Bean, 1985; Narahashi, Tsunoo & Yoshii, 1987). (See also Matteson & Armstrong (1986) for a classification of the channels.) Transient type Ca^{2+} channels open at membrane potentials between -50 and -35 mV (but see Fedulova *et al.* (1985) for an exception), whereas sustained channels open at more positive membrane potentials (> -30 mV). The different types of Ca^{2+} channels are also distinguished toxicologically and pharmacologically (reviewed by Miller, 1987; Narahashi *et al.* 1987).

Adult frog skeletal muscles are preparations commonly used for electrophysiological studies of voltage-sensitive Ca^{2+} channels, where only sustained type channels are usually seen (Stanfield, 1977; Sánchez & Stefani, 1978; Almers & Palade, 1981; but see Cota & Stefani, 1986; Arreola, Calvo, García & Sánchez, 1987). In contrast, in cell-cultured rat skeletal muscle cells, at least two types of voltage-sensitive Ca^{2+} channels, a transient type and a sustained type, are reported (Cognard, Lazdunski & Romey, 1986; Beam, Knudson & Powell, 1986). The transient type is insensitive to dihydropyridines and opens at more negative membrane potentials than the other. Action potential recording in cultured chick skeletal muscle cells suggests that the avian skeletal muscles also have two types of Ca^{2+} channels (Kano, Wakuta & Satoh, 1987). These results suggest that two types of Ca^{2+} channels may exist and play a role during normal development. We have tested this hypothesis in the studies presented here.

Changes in some properties of mammalian skeletal muscle which seem important for maturation of the neuromuscular system occur within a few weeks after birth. Polyneuronal innervation disappears, and an adult pattern of end-plate innervation is established within 16–18 days after birth (Redfern, 1970). Extrajunctional acetylcholine (ACh) receptors are eliminated (Diamond & Miledi, 1962; Steinbach, Merlie, Heinemann & Bloch, 1979), and the open time of junctional ACh channels shortens to a value characteristic of adult channels during the first 2 weeks after birth (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980). Differentiation in muscle fibre types also occurs in this period (reviewed by Close, 1972).

In the present paper, we report that in mouse skeletal muscle current through the transient Ca^{2+} channels disappears during post-natal development, whereas current

through the sustained type is increased. We also describe effects of denervation and tissue culture on these Ca^{2+} channels.

A brief report that Ca^{2+} current increases in rat skeletal muscle during post-natal development has been published independently in abstract form (Knudson, Jay & Beam, 1986) while our work was in progress.

METHODS

Preparation. Male and female ICR mice (Charles River, Japan) aged between 1 (body weight, $1{\cdot}5 \pm 0{\cdot}03$ g) and 30 days (23 ± 3 g) were used. Our method for isolating single skeletal muscle fibres was similar to that described by Bekoff & Betz (1977) for isolating adult rat digital muscles. After the mice had been killed by rapid decapitation, flexor digitorum brevis muscles were removed from right and left hindlimbs under a dissecting microscope. Dissected muscles were rinsed in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (2·7 mM-KCl, 1·5 mM-KH_2PO_4, 136·9 mM-NaCl, 15·2 mM-Na_2HPO_4, pH 7·4). The muscles were then incubated in 0·5 ml Ca^{2+}- and Mg^{2+}-free Eagle's minimum essential medium (MEM; prepared from vitamin and amino acid solutions, Gibco) containing 0·3% collagenase type I (Sigma). For mice younger than 10 days old, 0·15% collagenase solution was used instead of 0·3%. The muscles were incubated at 37 °C for 3 h under an atmosphere of 5% CO_2–95% air at 100% humidity. After the incubation, muscles were washed with recording medium (see below), and single muscle fibres were obtained by gentle trituration.

Denervation. Denervation was performed on 8- or 17-day-old mice under ether anaesthesia. When the tibial nerve was cut, an incision was made on the medial surface of the left knee and 1 mm of the nerve was removed. The sciatic nerve was cut at the left thigh near the pelvis. The wound was disinfected. The contralateral side was left intact. Every 5th day after the operation, the wounds were carefully reopened under ether anaesthesia, and any sign of regenerating nerve was removed.

Tissue culture. The muscles isolated from 8- or 17-day-old mice were used. After the incubation in the collagenase solution for 3 h, muscles were rinsed in Ca^{2+}- and Mg^{2+}-free MEM and then suspended in culture medium which consisted of Dulbecco–Vogt's modified Eagle essential medium containing 10% horse serum (Flow Laboratories), 5% newborn calf serum (Gibco), penicillin (50 U/ml) and streptomycin (50 μg/ml). The sera were heat-inactivated. Single fibres obtained by trituration were seeded in 35 mm plastic dishes (Falcon) containing 1 ml culture medium on a one muscle per dish basis. The cells were incubated under the atmosphere described above for the collagenase treatment. One-half of the culture medium was changed every 2 days. At the end of a culture period, muscle fibres were rinsed in the recording medium. The cultured muscle fibres, which have cylindrical shapes and clear striations, were easily distinguished from flat and highly branched myotubes formed from satellite cells in culture.

Voltage clamp recordings. The muscle fibres used in the experiments ranged between 63 and 625 μm in length and 10 and 20 μm in diameter. In addition to intact muscle fibres, cell fragments formed during the trituration process that did not contract in the Ba^{2+}-containing recording medium were used. A plastic dish containing isolated muscle fibres in 1 ml recording medium was mounted on the stage of an inverted phase-contrast microscope. The recording medium consisted of 30 mM-$Ba(OH)_2$, 90 mM-tetraethylammonium (TEA) hydroxide (Sigma), 10 mM-TEA chloride (Sigma), 140 mM-methanesulphonate (Aldrich), 30 mM-glucose and 10 mM-3-(*N*-morpholino)propanesulphonic acid (MOPS, adjusted to pH 7·4 by methanesulphonate). Barium-free perfusion medium consists of 135 mM-TEA hydroxide, 10 mM-TEA chloride, 125 mM-methanesulphonate, 30 mM-glucose and 10 mM-MOPS (pH 7·4). Tetrodotoxin (TTX, Sankyo) was added to the recording media at a final concentration of 20 μM in all the recordings. Recordings were performed at room temperature (20 ± 2 °C).

The voltage clamp was based on the one-pipette gigaseal, whole-cell recording technique (Hamill *et al.* 1981). Glass microtubes (1·5 mm o.d. made of soft glass, Terumo, Japan) were pulled in two steps and fire-polished without extra coating. The pipette was filled with 110 mM-caesium aspartate, 25 mM-CsF, 15 mM-CsCl and 10 mM-MOPS solution (adjusted to pH 7·2 with CsOH). The internal pipette medium was filtered through 0·45 μm filters (Toyo, Japan) before filling the micropipettes. The tip resistances of the pipettes were between 0·5 and 0·9 MΩ in the recording

medium. The pipette was allowed to form a gigaohm seal on the cell membrane, and the membrane was immediately ruptured with a pulse of suction to create whole-cell recording conditions with continuity between the pipette filling solution and the cell cytoplasm.

A List EPC-7 patch clamp system (List Electronic, F.R.G.) was used for voltage clamp recordings without bridges between electrodes and the recording solutions. The series resistance of the pipette and the cell was compensated by an internal feed-back circuit. Linear leakage current was subtracted with an analog subtractor. Currents were digitized and stored on a floppy disc with

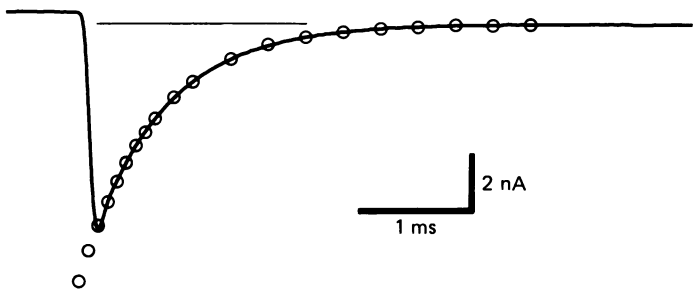

Fig. 1. Capacitive transient current of the cell membrane in a mouse skeletal muscle fibre. The fibre was isolated from the flexor digitorium brevis muscle of a 25-day-old mouse. The capacitive transient current was recorded by hyperpolarizing the cell membrane to -100 mV from a holding potential of -90 mV. The cut-off frequency of the filter was 19 kHz. Total capacitance of the cell membrane calculated from the charge moved was 545 pF. The diameter and length of the fibre were 15 and 425 μm, respectively. Circles show the fit by a single-exponential curve with a decay time constant of 656 μs.

an NEC PC9801 computer. Current records were filtered through a four-pole Bessel filter cutting off (-3 dB) at 3·1 kHz and sampled at 400 μs intervals. The cells were maintained at a holding potential of -90 mV. For measurement of voltage-dependent activation of Ca^{2+} channel current, the membrane was depolarized with test pulse potentials from -60 to $+50$ mV in 10 mV steps at 10 s intervals. For measurement of voltage-dependent inactivation of transient Ca^{2+} channels, the holding potential was varied from -150 to -40 mV for 10 s, followed by a test pulse to -30 mV for 100 ms to elicit Ca^{2+} channel current. For measurement of voltage-dependent inactivation of sustained Ca^{2+} channels, the membrane potential was varied from -130 or -110 mV to -20 or 0 mV for 10 s, followed by a test pulse to $+20$ mV for 100 ms to elicit Ca^{2+} channel current.

Amplitudes of transient and sustained Ca^{2+} channel currents were evaluated from recordings made within 4 min after making a seal. Regression curves in the Figures were obtained by the least-squares method. Statistical data were analysed by Student's t test.

Capacitive transient current of muscle fibres. Capacitive transient currents of muscle fibres were measured by hyperpolarizing the cell membrane from -90 mV to -100 mV without using the feed-back circuit for series resistance compensation. The membrane capacitance was calculated from the area (charge) under the capacitive transient current. The capacitance of the muscle cells ranged between 112 and 1030 pF. When referred to the apparent surface area of the fibre, taken as a cylinder, the specific capacitance was $2{\cdot}97 \pm 0{\cdot}96$ $\mu F/cm^2$ (S.D., $n = 151$) for innervated fibres of 1- to 30-day-old animals, indicating that the cells have 3 times more surface area than is apparent under the photo-microscope. No statistical difference in the specific capacitance was observed among fibres from 1- to 5-, 6- to 10-, 11- to 20- and 21- to 30-day-old animals ($P > 0{\cdot}1$). Also, the specific membrane capacitance of the denervated fibres was not different from that of the innervated ones of the same age ($P > 0{\cdot}1$). Although the skeletal muscle fibre is a long and thin preparation with T-tubules, the decay of the capacitive transient current could be fitted well by a single-exponential function (Fig. 1), with the decaying time constant ranging between 0·16 and 2·2 ms (mean $\pm$ S.D., $0{\cdot}87 \pm 0{\cdot}43$, $n = 151$). The series resistance of the pipette and the cell, which

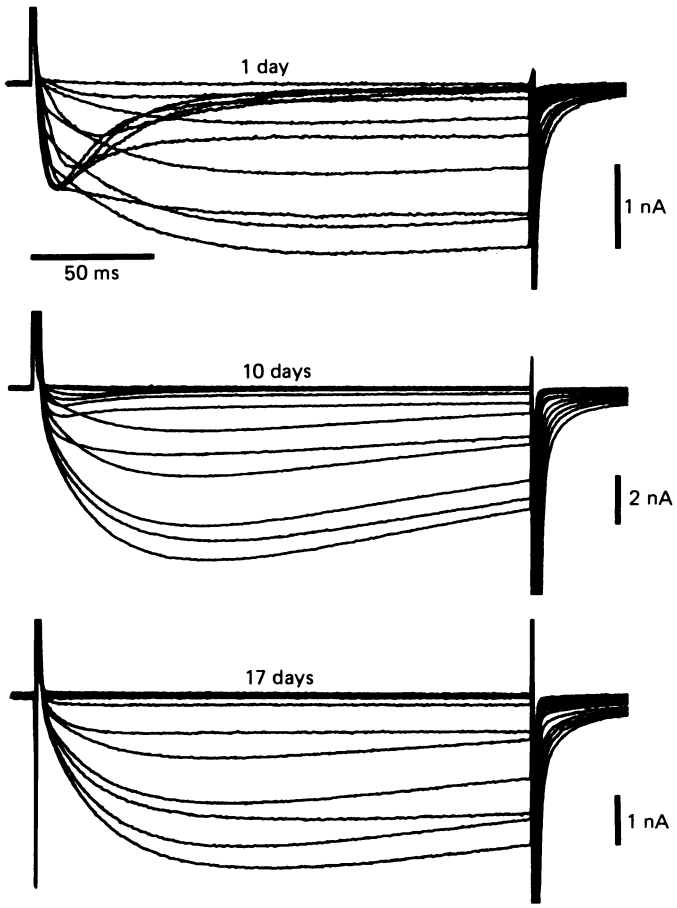

Fig. 2. Families of Ca^{2+} channel currents in fibres isolated from 1-, 10- and 17-day-old mouse skeletal muscles under voltage clamp. The extracellular recording medium contained 30 mM-Ba^{2+} as described under Methods. The fibres were maintained at a holding potential of −90 mV. The families of currents were elicited by test pulses from −60 to +50 mV in 10 mV intervals applied at 10 s time intervals. Total capacitance of the cell membrane was 251, 553 and 334 pF for the 1-, 10- and 17-day-old fibres, respectively. The time scale is common for the three sets of records.

was calculated by dividing the time constant of the uncompensated capacitive transient by the membrane capacitance, was $2{\cdot}09 \pm 1{\cdot}04$ (S.D., $n = 151$) MΩ. The positive feed-back circuit for the compensation reduced the apparent series resistance to $\frac{1}{7}$ to $\frac{1}{2}$ of the uncompensated value, which seemed satisfactory for the purpose of our present investigation.

The specific currents given in the Figures are referred to the membrane capacitance rather than the visible surface area.

RESULTS

Changes in calcium channel currents during post-natal development

Figure 2 shows families of inward currents in fibres isolated from flexor digitorum brevis muscles of 1-, 10- and 17-day-old mice. The recording medium contained

30 mM-Ba^{2+} (see Methods). Similar currents were also observed in medium containing 30 mM-Ca^{2+} in place of Ba^{2+} (data not shown). However, because the presence of Ca^{2+} caused the fibres to contract strongly upon membrane depolarization, which often disturbed current recordings, and because Ca^{2+} is reported to inactivate Ca^{2+} channels (reviewed by Hagiwara, 1983), all the recordings described in this paper were performed in Ba^{2+}-containing medium. Contractions of muscle fibres in

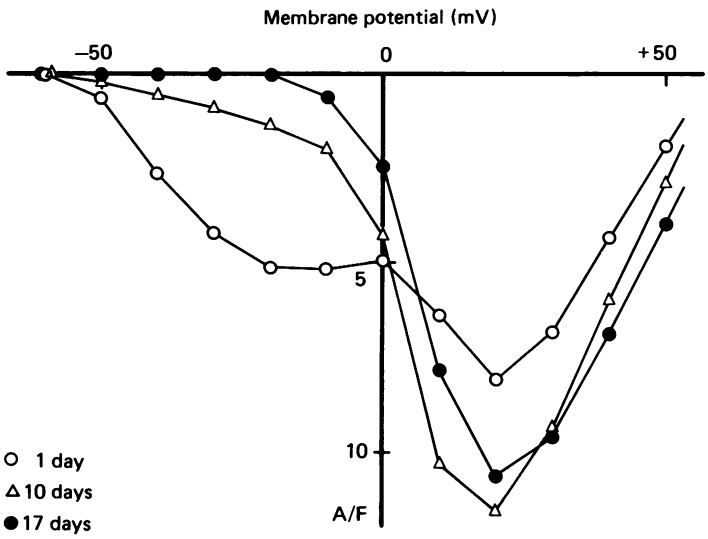

Fig. 3. Current–voltage relationships of the 1-, 10- and 17-day-old muscle fibres shown in Fig. 2. The specific current was calculated by dividing the maximum current obtained during the 200 ms test pulse by the total capacitance of the fibre and plotted on the ordinate. The abscissa indicates the test pulse potential. One- (○), 10- (△) and 17-day-old fibres (●).

response to depolarizing pulses were present but very weak in 30 mM-Ba^{2+} solution. In 1-day-old fibres, the inward currents have two components (Fig. 2, top). The first component is a transient current ($I_{transient}$), which activates at a membrane potential more positive than −50 mV and inactivates quickly. The second component is a sustained current ($I_{sustained}$), which activates more slowly than the first type at a membrane potential more positive than −20 mV. The two current components in the freshly isolated 1-day-old fibres were similar to those described for Ca^{2+} currents in rat skeletal muscles in cell culture (Cognard *et al.* 1986; Beam *et al.* 1986).

On day 10 after birth, the inward currents still had two components, but the transient one was smaller in amplitude (Fig. 2, middle). By day 17, the transient component had disappeared in all the fibres studied (Fig. 2, bottom).

Both types of current were Ba^{2+} currents carried through Ca^{2+} channels for the following reasons: (1) there were no ions which could carry significant inward currents in the recording medium except Ba^{2+}; (2) Na^+ channels were blocked by 20 μM-TTX, a concentration high enough to block even TTX-insensitive Na^+ channels (Gonoi, Sherman & Catterall, 1985), and K^+ channels were blocked by external TEA and by internal Cs^+ (see Methods); (3) the time constant of

inactivation of $I_{transient}$ was about 10 times longer than that of Na^+ currents in fibres recorded under similar conditions; (4) both currents were blocked by millimolar addition of Co^{2+} or Cd^{2+} to the bath; (5) both currents decreased when the concentration of Ba^{2+} in the recording medium was reduced by perfusing the bath with Ba^{2+}-free medium (data not shown).

Figure 3 shows the voltage dependence of maximum Ca^{2+} channel currents obtained during a 200 ms test pulse in the 1-, 10- and 17-day-old muscle fibres shown

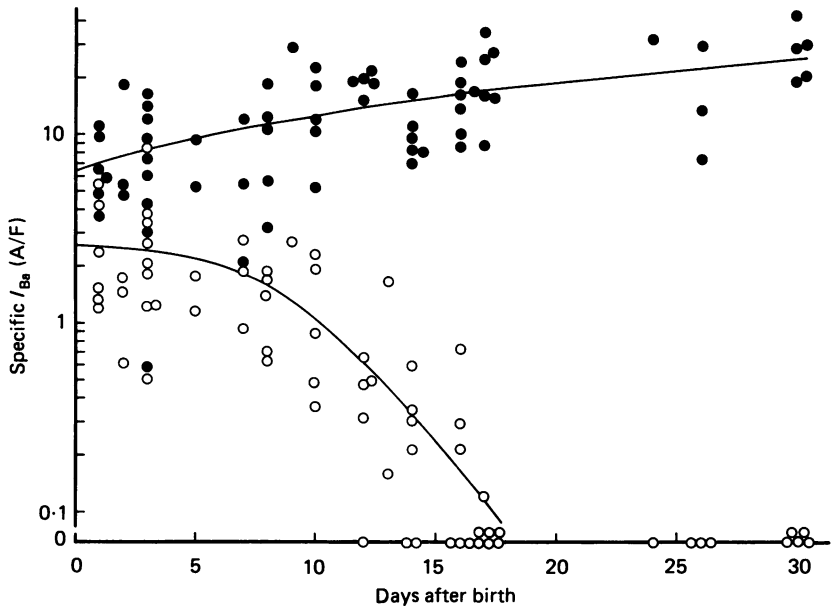

Fig. 4. Changes in the amplitudes of the transient and sustained Ca^{2+} channel currents during post-natal development. For evaluating amplitudes of $I_{transient}$ and $I_{sustained}$, the specific Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels elicited by -30 mV (○) and $+20$ mV test pulses (●) were plotted, respectively. The two continuous lines in the Figure were obtained by the least-squares method assuming a linear increase of $I_{sustained}$ ($I = aT + b$; where I and T are current amplitude and days after birth, respectively, $a = 0{\cdot}61$ A F^{-1} day^{-1}, $b = 6{\cdot}5$ A/F), and an equation, $I = c/[1 + \exp\{(T-d)/e\}]$ for the decrease of $I_{transient}$ (where $c = 2{\cdot}7$ A/F, $d = 8{\cdot}8$ days and $e = 2{\cdot}6$ days).

in Fig. 2. In the 1-day-old fibre, the current–voltage curve shows two peaks, one at -20 mV and the other one at $+20$ mV, which correspond to the two components of the currents shown in Fig. 2: the low-threshold $I_{transient}$ and the high-threshold $I_{sustained}$, respectively (○). In the 10-day-old fibre, the low-threshold component decreased in its amplitude, while the high-threshold one increased (△). In the 17-day-old fibre, the low-threshold $I_{transient}$ had decreased further and become undetectable (●). The voltage dependence in activating $I_{sustained}$ is similar among the three fibres of the different ages. In most of the fibres which had developed *in situ*, maximal $I_{sustained}$ was observed at a test pulse to $+10$ or $+20$ mV. The reversal potential of $I_{sustained}$ was more positive than $+50$ mV in fibres from all ages studied.

In Fig. 4, post-natal changes in current amplitudes for $I_{transient}$ (○) and $I_{sustained}$

(●) are plotted on a logarithmic scale on the ordinate. The specific $I_{transient}$ and $I_{sustained}$ were evaluated from the maximum currents at −30 and +20 mV test pulses, respectively, as described in the Methods. At these membrane potentials, the currents can be recorded individually without cross-contamination, because at a test pulse to −30 mV $I_{sustained}$ is not elicited, and at a test pulse to +20 mV, $I_{sustained}$ takes more than 70 ms to reach its maximum, by which time $I_{transient}$ is almost

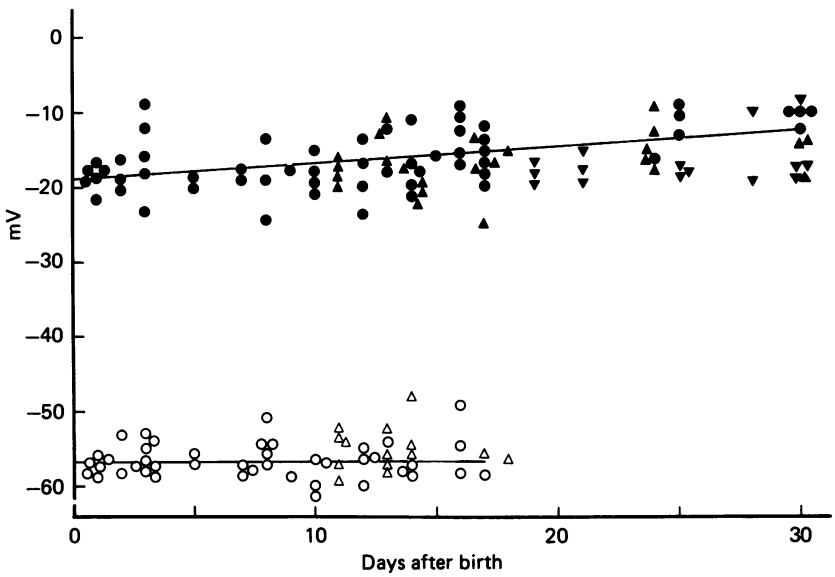

Fig. 5. Changes in activation threshold potentials for the transient and sustained Ca^{2+} channel currents in innervated and denervated mouse muscle fibres. The threshold potentials for $I_{transient}$ (open symbols) and $I_{sustained}$ (filled symbols) were obtained from current–voltage curves as potentials which elicit 5% of the maximum current at −30 and +20 mV test pulses, respectively. Innervated (circles), 8th-day-denervated (triangles) and 17th-day-denervated fibres (inverted triangles). Note that $I_{transient}$ of these fibres disappeared after 17 post-natal days. The bottom and top lines are linear regression curves for $I_{transient}$ and $I_{sustained}$ of innervated fibres, respectively ($V = aT + b$; where V and T are the threshold potential and days after birth, respectively, $a = 0{\cdot}02$ mV/day, $b = -56{\cdot}9$ mV for $I_{transient}$. The regression coefficient, a, is not significantly large in comparison to the sample standard deviation ($P > 0{\cdot}1$) and $a = 0{\cdot}19$ mV/day, $b = -18{\cdot}6$ mV for $I_{sustained}$. The value of a is significantly large ($P < 0{\cdot}001$).

completely inactivated and negligible (see Figs 2 and 9). The specific $I_{transient}$ and $I_{sustained}$ varied from fibre to fibre even at the same age. The mean of the specific $I_{transient}$ on day 1 after birth was $2{\cdot}7 \pm 1{\cdot}7$ ampere/farad (A/F; S.D., $n = 6$), ranging between 1·19 and 5·44 A/F. Although it was not quantified, we had the impression that among randomly selected fibres, long fibres, with well-developed striations had a smaller $I_{transient}$. The value of $I_{transient}$ decreased progressively with age and disappeared by day 17. For estimating the time course of the disappearance, we introduced the equation $I = c/[1 + \exp\{(T-d)/e\}]$ and applied the least-squares method to calculate the constants. (For details, see the legend to Fig. 4.) The curve fits the data well, showing that the specific $I_{transient}$ decreased to half by about day 9 after birth (Fig. 4, bottom curve).

On the other hand, the specific $I_{sustained}$ increased during the post-natal period from 6·9±2·7 A/F (S.D., $n = 6$) on day 1 to 27·7±8·5 A/F ($n = 5$) on day 30 after birth. The increase was 4-fold in 30 days. A regression curve for the specific $I_{sustained}$ is also drawn in Fig. 4 (top curve). Thus the two current components have different fates during post-natal development.

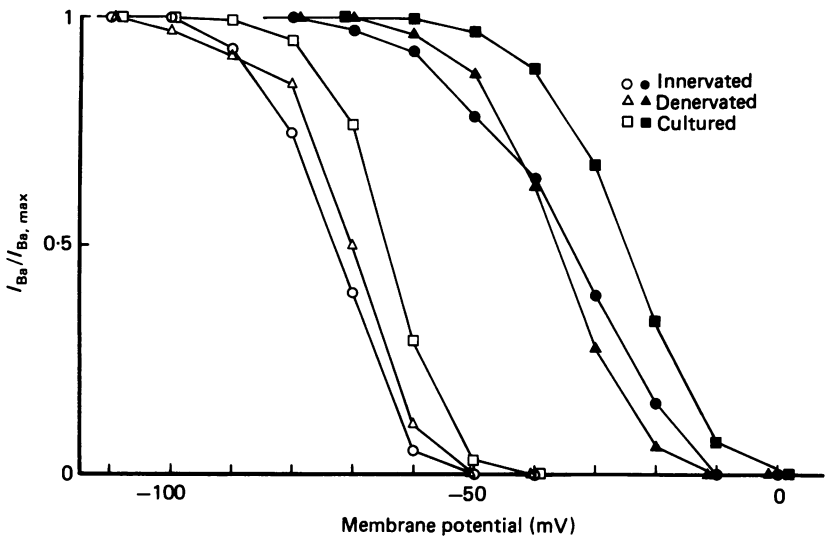

Fig. 6. Voltage-dependent inactivation of the transient and sustained Ca^{2+} channel currents in innervated, denervated and cultured muscle fibres. Muscle fibres were maintained at the holding potential indicated on the abscissa for 10 s and stimulated with a test pulse to −30 or +20 mV to obtain the inactivation curve for $I_{transient}$ and $I_{sustained}$, respectively. For details, see Methods. Open symbols: $I_{transient}$ in innervated 8-day-old (circles), 8th-day-denervated 11-day-old (triangles), and 8th-day-isolated 3-day-cultured fibres (11-day-old, squares). Filled symbols: $I_{sustained}$ in innervated 24-day-old (circles), 8th-day-denervated 24-day-old (triangles), and 17th-day-isolated 11-day-cultured (28-day-old, squares) fibres. Note that the inactivation curves of $I_{transient}$ and $I_{sustained}$ in the cultured fibres are shifted in the positive direction from the corresponding curves of the currents in the innervated and denervated fibres, respectively.

Changes in activation threshold and inactivation potentials of calcium currents during the post-natal period

The disappearance of $I_{transient}$ could be explained if the threshold potential for activating $I_{transient}$ shifted in the positive direction so that $I_{transient}$ became hidden under the large $I_{sustained}$ or if the inactivation curve of $I_{transient}$ shifted in the negative direction so that the currents were inactivated at the holding potential (−90 mV) used for the recordings. Studying the changes in the threshold potentials of the Ca^{2+} channel currents is also interesting because a negative shift in voltage-dependent activation of Na^+ channels during development was suggested in rat skeletal muscle (Gonoi *et al.* 1985).

We calculated the threshold potentials of $I_{transient}$ and $I_{sustained}$ from current–

voltage curves as potentials which elicited 5% of the maximum currents at −30 and +20 mV test pulses, respectively. To evaluate the threshold potential of $I_{sustained}$, the current at the end of the 200 ms test pulse, at which time $I_{transient}$ was inactivated, was used.

Figure 5 shows changes of the threshold potentials for $I_{transient}$ (○) and $I_{sustained}$ (●) during the post-natal period. The threshold potential for $I_{transient}$ changed little in the

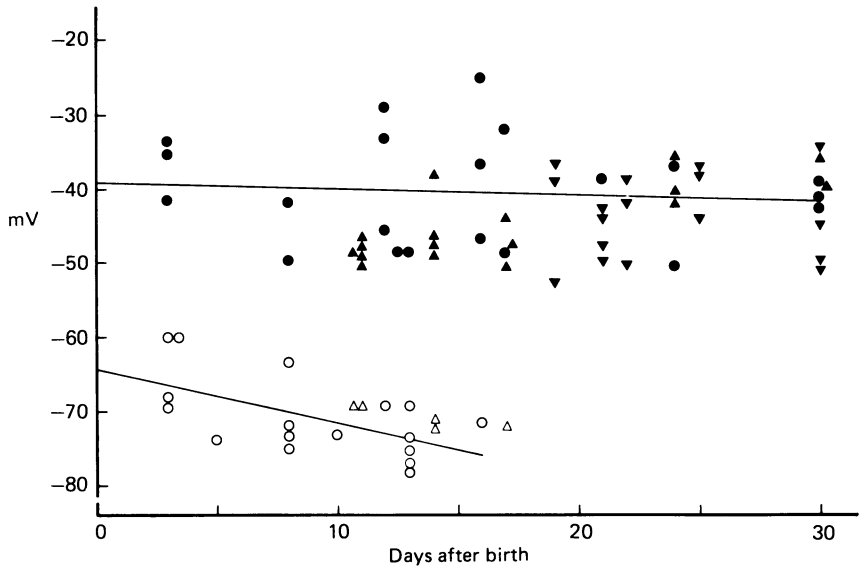

Fig. 7. Changes in the half-inactivation potential of $I_{transient}$ and $I_{sustained}$ in innervated and denervated mouse muscle fibres during post-natal development. The half-inactivation potentials for $I_{transient}$ (open symbols) and $I_{sustained}$ (filled symbols) were obtained as described in Methods and the legend of Fig. 6. Innervated (circles), 8th-day-denervated (triangles) and 17th-day-denervated fibres (inverted triangles). Note that $I_{transient}$ of these fibres disappeared after 17 post-natal days. The bottom and top lines are linear regression curves for $I_{transient}$ and $I_{sustained}$, respectively ($V = aT + b$; where V and T are the half-inactivation potential and days after birth, respectively, $a = -0{\cdot}67$ mV/day, $b = -65{\cdot}0$ mV for $I_{transient}$ and $a = -0{\cdot}10$ mV/day, $b = -38{\cdot}9$ mV for $I_{sustained}$).

17 post-natal days during which $I_{transient}$ was observed. The mean for the innervated fibres from day 1 to 17 was $-56{\cdot}7 \pm 2{\cdot}5$ mV (S.D., $n = 41$). The threshold potential for $I_{sustained}$ shifted only a little in the positive direction (by 5·8 mV as judged by the regression curve in the Figure).

Figure 6 shows typical examples of inactivation curves for $I_{transient}$ and $I_{sustained}$ from an 8-day-old fibre (○ and ●, respectively). The inactivation curve for $I_{transient}$ is located at a potential 40 mV more negative than that for $I_{sustained}$, while the steepness of the curves is similar.

The developmental changes in the half-inactivation potentials for $I_{transient}$ and $I_{sustained}$ are plotted in Fig. 7 (○ and ●, respectively). The half-inactivation potential of $I_{transient}$ shifted in a negative direction from $-67{\cdot}0 \pm 5{\cdot}0$ (S.D.) mV on day 3 to $-73{\cdot}7 \pm 4{\cdot}0$ mV on day 13 (6·7 mV shift; statistically significant, $P < 0{\cdot}025$). The steepness of the inactivation curve did not change significantly during post-natal

development (data not shown). Figure 6 shows that when the half-inactivation potential of $I_{transient}$ is −73 mV, only 7% of the transient channels are inactivated at the holding potential of −90 mV. The negative shift in the inactivation potential contributes to the reduction of $I_{transient}$ by less than 10%. Therefore, the disappearance of $I_{transient}$ is not explained by shifts in activation threshold potential or inactivation potential during development.

The mean value of the half-inactivation potential for $I_{sustained}$ changed little (< 3 mV) in the 30 post-natal days.

Changes in activation and inactivation times of the currents during the post-natal period

The disappearance of $I_{transient}$ could also be explained if the time required to activate transient channels became longer and/or the time required to inactivate them became shorter, so that even if channels existed in the membrane they would not produce current distinguishable from $I_{sustained}$. We tested these hypotheses by examining the rise (t_{rise}) and fall (t_{fall}) times for $I_{transient}$ to go respectively from 25 to 75% and 75 to 25% of the peak amplitude recorded at −30 mV test pulse (Fig. 8*A*, left). The regression curves obtained as described in the Figure legend show that t_{rise} and t_{fall} of $I_{transient}$ became only 56 and 27% shorter in the 17 post-natal days (from 4·8 to 2·6 ms and from 38·7 to 28·4 ms), respectively (Fig. 8*B*, ○ and △, respectively). The results show that both the activation and inactivation processes of the transient channels became slightly faster during development. However, because the activation process speeded up more than the inactivation process did, the kinetic changes cannot account for the disappearance of $I_{transient}$.

The t_{rise} for $I_{sustained}$ was similarly evaluated (Fig. 8*A*, right), and it became 39% shorter (from 28·5 to 17·5 ms) in the 30 post-natal days (Fig. 8*B*, ●).

Effects of denervation on the calcium channel currents

We studied effects of denervation on the Ca^{2+} channel properties by cutting either the sciatic or tibial nerves on day 8 or 17 after birth. Fibrillation was observed in every denervated muscle isolated for the recordings. Figure 9 (left column) shows two examples of Ca^{2+} channel currents, $I_{transient}$ in an 11-day-old muscle fibre that had been denervated on day 8 (top left) and $I_{sustained}$ in a 24-day-old muscle fibre that had been denervated on day 8 (bottom left). The values of $I_{transient}$ and $I_{sustained}$ in fibres denervated for 2–22 days were similar to those in innervated fibres at the corresponding ages. Current–voltage relationships of $I_{transient}$ and $I_{sustained}$ of the denervated fibres are shown in Fig. 10 (△ in the left and right panels, respectively). The two current–voltage curves for denervated fibres are similar to those for innervated fibres of the corresponding ages.

Post-natal changes of the specific Ca^{2+} channel currents in the 8th- and 17th-day-denervated fibres are plotted in Fig. 11. The dashed lines in the Figure are the regression curves obtained for $I_{transient}$ and $I_{sustained}$ for the innervated fibres in Fig. 4. The specific $I_{transient}$ in the 8th-day-denervated fibres decreased with a similar time course to that of innervated fibres, and had become undetectable by day 17 after birth (Fig. 11*A*, ○). Denervation on day 17 never developed a detectable $I_{transient}$ for up to 13 days after denervation (Fig. 11*B*, ○), just as in innervated fibres.

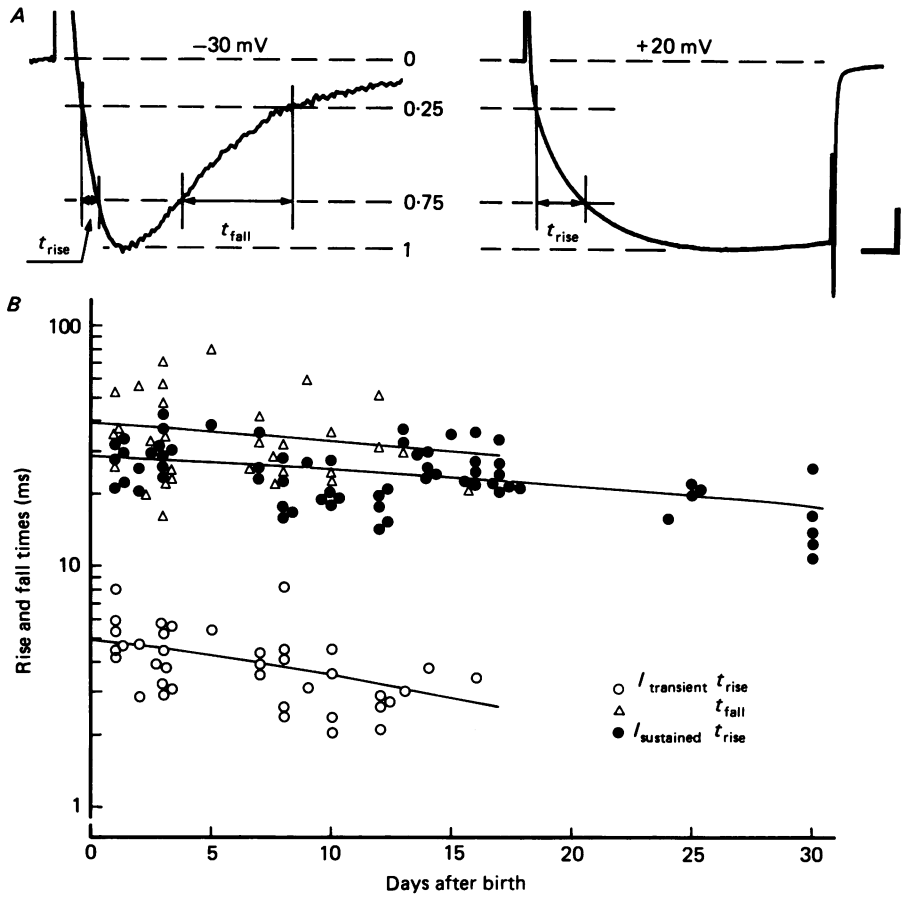

Fig. 8. Rise and fall times of $I_{transient}$ and $I_{sustained}$. *A*, the times required for activating the transient and sustained Ca^{2+} channels were evaluated as the rise times (t_{rise}) of the currents from 25 to 75% of the corresponding maximums (left and right). The time required for inactivating the transient channels was evaluated as the fall time (t_{fall}) from 75 to 25% of the maximum (left). The $I_{transient}$ and $I_{sustained}$ were elicited at a test pulse to −30 and +20 mV, respectively. The recordings shown are from 8 (left) and 13-day-old fibres (right), respectively. Calibration: 200 pA, 4·2 ms and 1 nA, 25 ms for the left and right records, respectively. *B*, changes in t_{rise} and t_{fall} of $I_{transient}$ (○ and △, respectively) or t_{rise} of $I_{sustained}$ (●) during post-natal development. The three continuous lines in the Figure are regression curves for the three parameters. $t = aT + b$; where t and T are t_{rise} (t_{fall}) and days after birth, respectively, $a = -0{\cdot}14$ ms/day, $b = 5{\cdot}0$ ms (t_{rise} of $I_{transient}$, the lower curve); $a = -0{\cdot}64$ ms/day, $b = 39{\cdot}3$ ms (t_{fall} of $I_{transient}$, the upper curve) or $a = -0{\cdot}38$ ms/day, $b = 28{\cdot}9$ ms (t_{rise} of the $I_{sustained}$, the middle curve).

The regression curves in Fig. 11*A* and *B* show that mean amplitudes of the $I_{sustained}$ in the 8th- and 17th-day-denervated fibres stayed at the levels on the days of denervation. The specific $I_{sustained}$ in innervated, 8th- and 17th-day-denervated fibres on day 30 after birth were $27{\cdot}7 \pm 8{\cdot}5$ A/F (S.D., $n = 5$), $15{\cdot}5 \pm 7{\cdot}4$ A/F ($n = 5$) and $15{\cdot}2 \pm 6{\cdot}8$ A/F ($n = 9$), respectively. Values of $I_{sustained}$ in 8th- and 17th-day-denervated fibres on day 30 were half of that in innervated fibres (statistically significant, $P < 0{\cdot}1$ and $P < 0{\cdot}025$, respectively).

Activation threshold potential and voltage-dependent inactivation of the currents in denervated muscles

Figure 5 shows activation threshold potentials of $I_{transient}$ and $I_{sustained}$ in 8th-day-denervated fibres (△ and ▲, respectively) and $I_{sustained}$ in 17th-day-denervated fibres (▼). There are no significant shifts relative to the corresponding parameters obtained from innervated fibres (○ and ●).

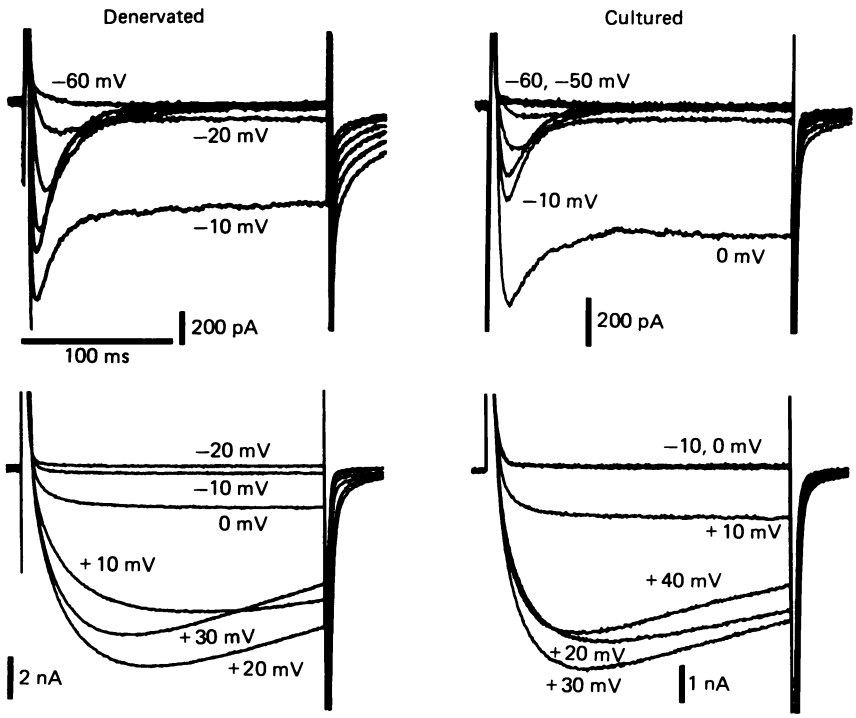

Fig. 9. Typical recordings of the transient (top panels) and sustained Ca^{2+} channel currents (bottom panels) in denervated (left column) and tissue-cultured (right column) mouse skeletal muscle fibres. The fibres were from 8th-day-denervated 11-day-old (top left), 8th-day-denervated 24-day-old (bottom left), 8th-day-isolated and 3-day-cultured (11-day-old, top right), and 8th-day-isolated and 15-day-cultured preparations (23-day-old, bottom right). The extracellular recording medium contained 30 mM-Ba^{2+}. Holding potential, −90 mV. The families of Ba^{2+} currents were elicited as described under Methods and in the legend of Fig. 2. The time scale is common for all the records. Note that in cultured fibres, a more positive test pulse potential was required to elicit $I_{transient}$ and $I_{sustained}$ than in the denervated fibres.

Examples of voltage-dependent inactivation curves for $I_{transient}$ and $I_{sustained}$ are shown in Fig. 6 for an 8th-day-denervated 11-day-old fibre (△ and ▲, respectively). We observed no significant differences in the voltage dependence of inactivation in $I_{transient}$ and $I_{sustained}$ between denervated and innervated fibres. Figure 7 shows that half-inactivation potentials of $I_{transient}$ and $I_{sustained}$ in 8th-day-denervated fibres (△ and ▲, respectively) and that of $I_{sustained}$ in 17th-day-denervated fibres (▼) are similar to the corresponding potentials in innervated fibres during the post-natal period.

Rise and fall times of the currents in denervated fibres

In fibres denervated for 2 or 3 days, the t_{rise} and t_{fall} of $I_{transient}$ and the t_{rise} of $I_{sustained}$ were 44–54% shorter than those for innervated fibres at the corresponding ages (statistically significant, $P < 0.05$ for all the parameters). Within 7 days after denervation, however, all these parameters returned progressively to the same levels as those found in innervated fibres (data not shown).

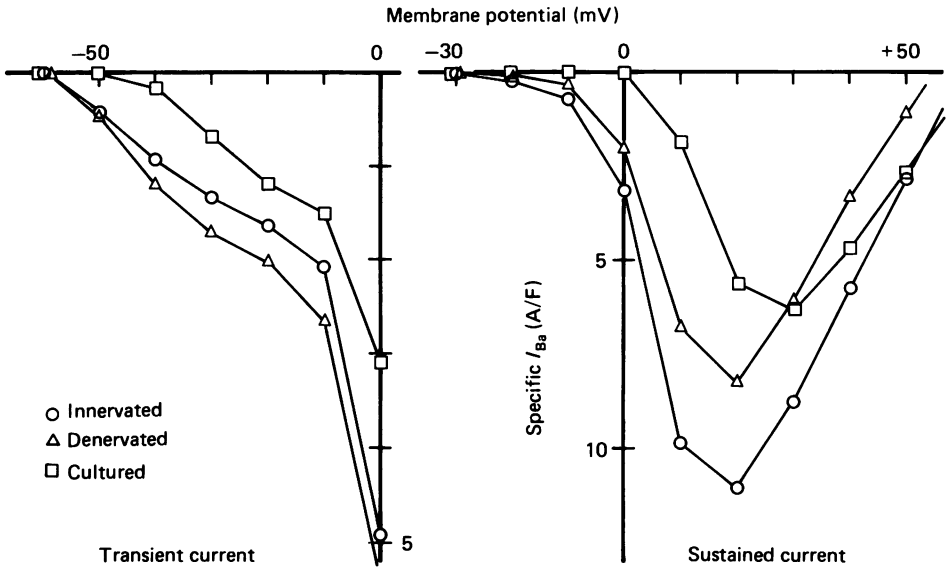

Fig. 10. The current–voltage relationships of $I_{transient}$ and $I_{sustained}$ in innervated, denervated or tissue-cultured mouse muscle fibres. Left: $I_{transient}$ was obtained from the early peak of Ba^{2+} current which was attained within 30 ms after starting a test pulse and declined quickly (see Figs 2 and 9). The specific current (current amplitude divided by total capacitance of the fibre) was plotted for innervated 8-day-old (○), 8th-day-denervated 11-day-old (△) and 8th-day-isolated 3-day-cultured (11-day-old, □) muscle fibres. Right: $I_{sustained}$ was obtained as current amplitude at the end of the 200 ms test pulse. Twenty-four-day-old (○), 8th-day-denervated 24-day-old, and 8th-day-isolated 15-day-cultured fibres (23-day-old, □). Note that the current–voltage relationships of $I_{transient}$ and $I_{sustained}$ in cultured fibres are shifted in the positive direction from the corresponding curves in innervated (and denervated) fibres.

Tissue culture-induced changes in the calcium channel properties

The recordings of $I_{transient}$ in a fibre isolated on the 8th day after birth and cultured for 3 days and $I_{sustained}$ in a fibre isolated on the 8th day and cultured for 15 days are shown in Fig. 9 (right column) as examples of Ca^{2+} channel currents in cultured fibres. The Ca^{2+} channel currents in cultured fibres were similar to those in innervated or denervated fibres, except that in cultured fibres a shift of approximately 10 mV to a more positive potential was often observed in the activation of ionic currents compared to innervated or denervated fibres. This can be seen more clearly in the current–voltage relationships of $I_{transient}$ and $I_{sustained}$ shown in Fig. 10.

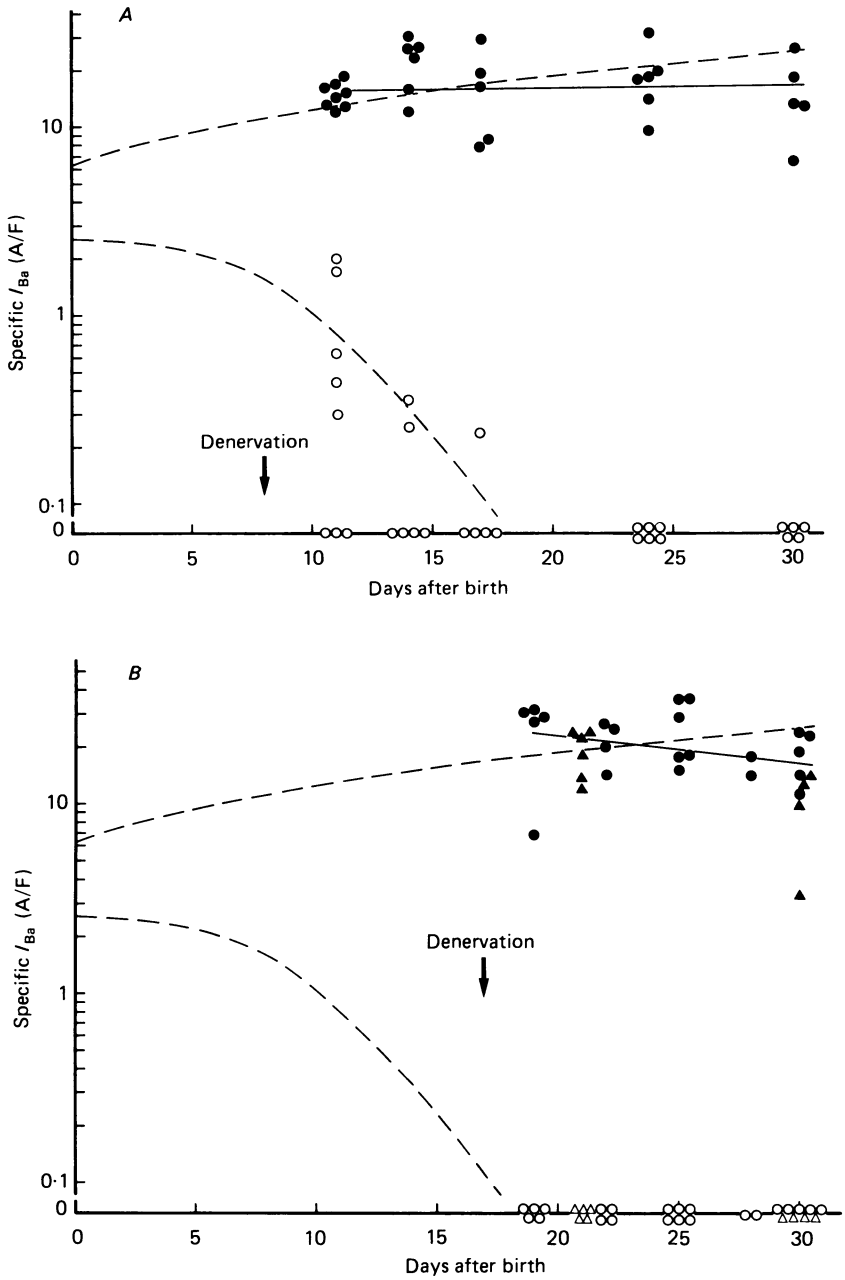

Fig. 11. Developmental changes in the amplitude of the transient and sustained Ca^{2+} channel currents in denervated fibres. *A*, the flexor digitorum brevis muscle was denervated on day 8 after birth by cutting the sciatic nerve as described in Methods. *B*, the muscle was denervated on day 17 by cutting the tibial nerve (circles) or the sciatic nerve (triangles). The specific $I_{transient}$ (open symbols) and $I_{sustained}$ (filled symbols) were plotted on the ordinate in each panel as described in Fig. 4. The continuous line in each panel is a linear regression line for $I_{sustained}$. $I = aT + b$, where $a = -0{\cdot}017$ A F^{-1} day^{-1}, $b = 17{\cdot}4$ A/F for *A* and $a = -0{\cdot}64$ A F^{-1} day^{-1}, $b = 35{\cdot}8$ A/F for *B*. The dashed lines are regression curves for the currents in innervated fibres as obtained in Fig. 4.

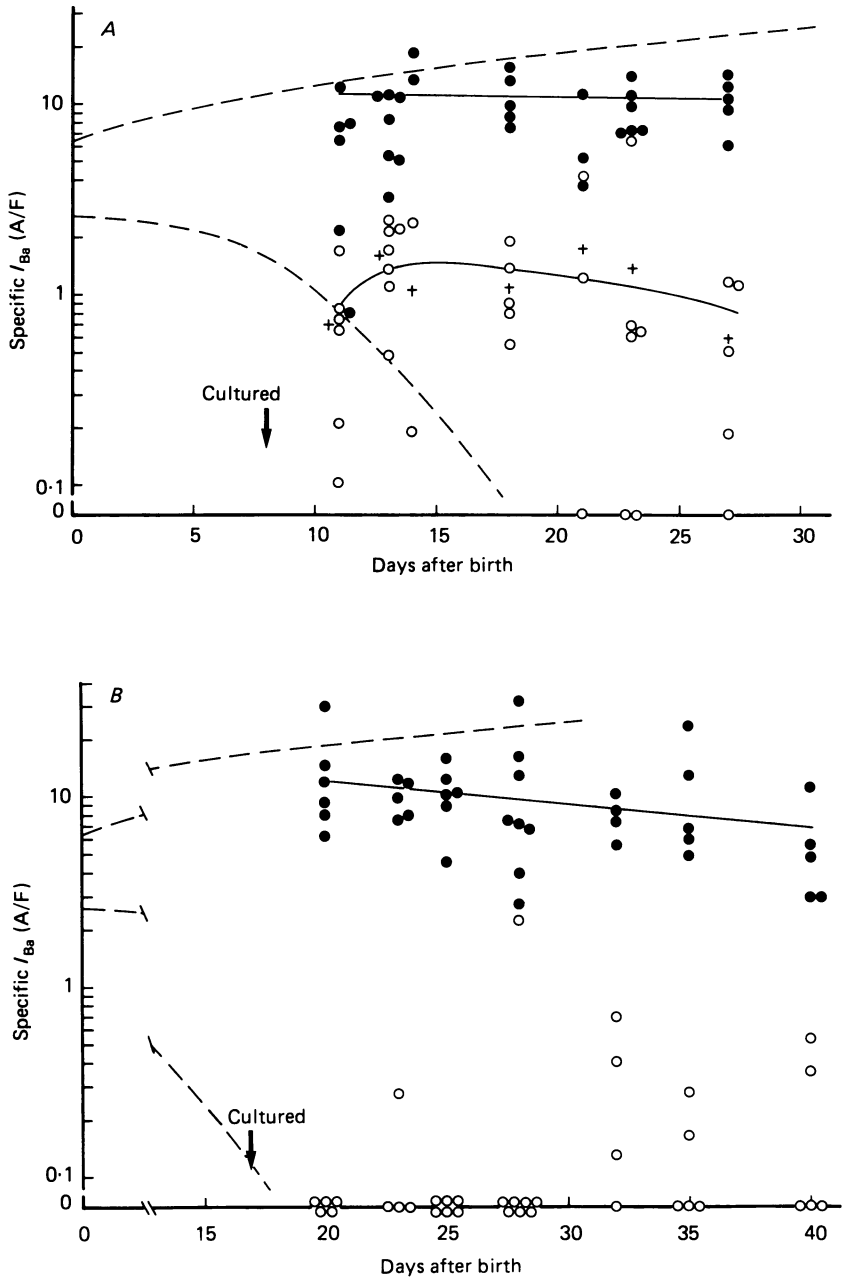

Fig. 12. Changes in the amplitudes of transient and sustained Ca^{2+} channel current in tissue-cultured skeletal muscle fibres. The muscle fibres were isolated on day 8 (*A*) or 17 (*B*) after birth and cultured as described in Methods. The specific $I_{transient}$ (○) and $I_{sustained}$ (●) were plotted. The continuous line at the top of each panel is a regression curve for $I_{sustained}$. $I = aT + b$; where $a = -0{\cdot}067$ A F^{-1} day^{-1}, $b = 11{\cdot}9$ A/F for *A* and $a = -0{\cdot}29$ A F^{-1} day^{-1}, $b = 18{\cdot}4$ A/F for *B*. The lower continuous curve in *A* is drawn by eye near the means of $I_{transient}$ which are indicated by crosses. The regression curves for the two types of currents in innervated fibres obtained in Fig. 4 are shown as dashed lines.

Figure 12 shows changes in the specific $I_{transient}$ and $I_{sustained}$ in cultured fibres. The $I_{transient}$ in 8th-day-isolated and cultured fibres stopped decreasing and stayed at about the same level for 3 weeks in culture, although we occasionally found fibres which had no detectable $I_{transient}$ (Fig. 12*A*). When the fibres were isolated on day 17, by which time $I_{transient}$ in all the innervated fibres disappeared (see Fig. 4), and were cultured thereafter, all but two fibres showed no $I_{transient}$ up to 11 days after starting the culture (28th day after birth, Fig. 12*B*).

The $I_{sustained}$ in cultured fibres stopped increasing or slightly decreased after isolation on day 8 or 17 (● in Fig. 12*A* and *B*). We observed a similar effect in denervated fibres (see Fig. 11).

The specific currents of the cultured fibres were evaluated at the same test pulse potentials used for innervated and denervated fibres (-30 and $+20$ mV for $I_{transient}$ and $I_{sustained}$, respectively). If we take the shifts in the current–voltage relationships described above into account, maximum $I_{transient}$ and $I_{sustained}$ in cultured fibres are at most 40 and 10% larger than the values plotted in Fig. 12, respectively. However, such underestimates should not affect the results qualitatively.

In fibres cultured for more than 5 days, the means of the activation potentials of $I_{transient}$ and $I_{sustained}$ were shifted in the positive direction by approximately 8 mV from the corresponding parameters for the innervated fibres ($-48{\cdot}4 \pm 3{\cdot}1$ mV, $n = 28$ *vs.* $-56{\cdot}7 \pm 2{\cdot}4$ mV, $n = 41$, and $-8{\cdot}1 \pm 5{\cdot}5$ mV, $n = 44$ *vs.* $-16{\cdot}3 \pm 4{\cdot}2$ mV, $n = 55$, respectively. The values are for mean ± S.D., $P < 0{\cdot}005$, in both cases. Also see Figs 9 and 10 for the shifts). A similar shift was also observed in the half-inactivation potential of $I_{sustained}$ ($-33{\cdot}0 \pm 7{\cdot}2$ mV, $n = 24$ *vs.* $-40{\cdot}6 \pm 7{\cdot}3$ mV, $n = 21$, $P < 0{\cdot}005$, see Fig. 6).

DISCUSSION

Disappearance of the transient calcium channel current during development

The present study shows that $I_{transient}$ in mouse flexor digitorum brevis muscle decreases progressively after birth and becomes undetectable by day 17, while $I_{sustained}$ increases 4-fold from day 1 to day 30. The disappearance of $I_{transient}$ during the development is *not* caused by the following effects: (1) shift in the activation threshold potential of $I_{transient}$ in the positive direction, (2) shift in the threshold potential of $I_{sustained}$ in the negative direction so that $I_{transient}$ is hidden under a large $I_{sustained}$, (3) shift in voltage-dependent inactivation of $I_{transient}$ to a more negative potential so that the functional transient channels on the membrane are inactivated at the holding potential of -90 mV used in the experiments, (4) slowing down in the activation process, or (5) speeding up of the inactivation process of the transient channels so that no detectable $I_{transient}$ flows before the inactivation occurs.

Actually, no appreciable shifts were observed in the activation threshold potentials of the transient and sustained channels with time after birth (see Fig. 5). The half-inactivation potential of the transient channels shifted in the negative direction by 7 mV in the post-natal period (see Fig. 7), but the negative shift does not account for the disappearance of $I_{transient}$, because (1) the shift caused only a small amount of inactivation in the transient channels at the holding potential used (see Fig. 6) and (2) stimulating the fibres older than 17 days with a test pulse to -30 mV from a very

negative holding potential of −150 mV did not elicit any detectable $I_{transient}$ (data not shown). The inactivation potential of the sustained channels did not change appreciably after birth. The time required for channel activation and inactivation was evaluated as t_{rise} and t_{fall}, respectively (see Fig. 8). There was a small decrease in the t_{rise} and t_{fall} of $I_{transient}$ and a small decrease of the t_{rise} of $I_{sustained}$ with time after birth. Because in $I_{transient}$, t_{rise} decreased in a similar or slightly larger ratio than t_{fall}, the decrease and disappearance of $I_{transient}$ during development are not explained by these data. The t_{rise} of $I_{sustained}$ at a test pulse to +20 mV was 5 times longer than that of $I_{transient}$ at a test pulse to −30 mV throughout the post-natal period studied.

What mechanisms could cause the disappearance of the transient calcium channel current?

The possible mechanisms which cause the disappearance of $I_{transient}$ may be divided into two categories: (1) a large reduction in the number of transient Ca^{2+} channels produced or incorporated into the cell membrane or an accelerated rate in removal of the channels from the membrane and (2) inhibition of the transient Ca^{2+} channel conductance or gating mechanisms by chemical modification. At the present stage, we consider the first mechanism more likely because when fibres were isolated from 17-day-old animals and cultured *in vitro*, more than 2 weeks were required for $I_{transient}$ to become detectable again, long enough for cellular proteins to be transcribed, translated and incorporated into the membrane. The culture conditions do not seem to accelerate the disappearance of the transient channels, because fibres isolated on day 8 and cultured showed $I_{transient}$ until 19 days in culture (27th day after birth). The disappearance of $I_{transient}$ is gradual, whereas chemical modification might be expected to affect all transient channels in a short time span, once the modifying agent is produced. However, the latter possibility should also be considered because studies on voltage-sensitive Ca^{2+} channels in cardiac and skeletal muscle and other cells show that the Ca^{2+} conductance of the channels is modulated by cyclic AMP-dependent phosphorylation (reviewed by Reuter, 1983; Armstrong & Eckert, 1987; Arreola *et al.* 1987). In either case the developmental disappearance of $I_{transient}$ seems to be a very slowly reversible process in the cells.

What triggers the disappearance?

The present results indicate that innervation is unnecessary for the disappearance of the functional transient Ca^{2+} channels during the development. Denervation on day 8 after birth, when the density of the functional transient Ca^{2+} channels had started to decline, did not prevent the further decrease of the channels. Denervation on day 17 after birth, when all the transient Ca^{2+} channels had disappeared, did not cause reappearance of channels up to 30 days after birth. On the other hand, denervation caused fibrillation of the muscles and suppressed the normal post-natal increase of $I_{sustained}$ in both cases. The behaviour of transient Ca^{2+} channels after denervation contrasts to that of Na^+ channels in adult skeletal muscles, in which TTX-insensitive Na^+ channels reappear within a few days after denervation (Redfern & Thesleff, 1971).

Signals which might trigger the disappearance of the functional transient channels remain obscure. The disappearance of the transient channels might be under

humoral influences, because fibres which were isolated on day 8 after birth and cultured for the next 2 weeks still had the transient Ca^{2+} channels. Thus by day 8 after birth, muscle fibres were not simply destined to lose their transient Ca^{2+} channels. The possibility exists that some signal (whose nature is unknown at present) from the surrounding environment is required to initiate the loss of transient Ca^{2+} channels.

Speculative roles of the transient calcium channels

Calcium is involved in many cellular events as a second messenger. Tetrodotoxin-sensitive Na^+ channels in rat skeletal muscle increase 10-fold in the first 25 days after birth (Harris & Marshall, 1973; Sherman & Catterall, 1982), and extrajunctional ACh receptors are eliminated in a few weeks after birth (Diamond & Miledi, 1962). A regulatory role for Ca^{2+} in controlling the numbers of TTX-sensitive Na^+ channels and ACh receptors in muscle cell membranes has been proposed (Birnbaum, Reis & Shainberg, 1980; Sherman, Chrivia & Catterall, 1985; Rubin, 1985; Bloch, 1986). Calcium ions are probably involved in post-natal changes of other properties regulated by muscular activity such as ACh channel open time (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Brenner, Lømo & Williamson, 1987) and contractile properties (Salmons & Sréter, 1976), and in the post-natal elimination of polyneuronal innervation (reviewed by Purves & Lichtman, 1980). Because the transient Ca^{2+} channels open at membrane potentials closer to the resting membrane potential than the sustained channels, we think the transient channels might have a role in muscle development. It will be interesting to see what developmental changes the transient Ca^{2+} channels reported in other tissues like cardiac, smooth muscle, pituitary and dorsal root ganglion undergo.

Voltage-dependent activation and inactivation of the two types of Ca^{2+} channels in cultured fibres were shifted to a more positive potential than those of channels in fibres isolated for acute experiments. In some but not all the cultured cells studied previously, a shift in the activation potential towards the negative direction was experienced during recordings (Fernandez, Fox & Krasne, 1984; Gonoi *et al.* 1985; Gonoi & Hille, 1987). The mechanisms for the shifts are unknown, but the observations suggest the voltage-dependent kinetics of ion channels studied in cultured cells have to be applied with care to cells *in situ*.

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REFERENCES

ALMERS, W. & PALADE, P. T. (1981). Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. *Journal of Physiology* **312**, 159–176.

ARMSTRONG, D. & ECKERT, R. (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 2518–2522.

ARREOLA, J., CALVO, J., GARCÍA, M. C. & SÁNCHEZ, J. A. (1987). Modulation of calcium channels of twitch skeletal muscle fibres of the frog by adrenaline and cyclic AMP. *Journal of Physiology* **393**, 307–330.

BEAM, K. G., KNUDSON, C. M. & POWELL, J. A. (1986). A lethal mutation in mice eliminates the slow calcium current in skeletal muscle cells. *Nature* **320**, 168–170.

BEAN, B. P. (1985). Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *Journal of General Physiology* **86**, 1–30.

BEKOFF, A. & BETZ, W. J. (1977). Physiological properties of dissociated muscle fibres obtained from innervated and denervated adult rat muscle. *Journal of Physiology* **271**, 25–40.

BIRNBAUM, M., REIS, M. A. & SHAINBERG, A. (1980). Role of calcium in the regulation of acetylcholine receptor synthesis in cultured muscle cells. *Pflügers Archiv* **385**, 37–43.

BLOCH, R. J. (1986). Loss of acetylcholine receptor clusters induced by treatment of cultured rat myotubes with carbachol. *Journal of Neuroscience* **6**, 691–700.

BRENNER, H. R., LØMO, T. & WILLIAMSON, R. (1987). Control of end-plate channel properties by neurotrophic effects and by muscle activity in rat. *Journal of Physiology* **388**, 367–381.

CLOSE, R. I. (1972). Dynamic properties of mammalian skeletal muscles. *Physiological Reviews* **52**, 129–197.

COGNARD, C., LAZDUNSKI, M. & ROMEY, G. (1986). Different types of Ca^{2+} channels in mammalian skeletal muscle cells in culture. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 517–521.

COTA, G. & STEFANI, E. (1986). A fast-activated inward calcium current in twitch muscle fibres of frog (*Rana montezume*). *Journal of Physiology* **370**, 151–163.

DIAMOND, J. & MILEDI, R. (1962). A study of fetal and new-born rat muscle fibres. *Journal of Physiology* **162**, 393–408.

FEDULOVA, S. A, KOSTYUK, P. G. & VESELOVSKY, N. S. (1985). Two types of calcium channels in the somatic membrane of new-born rat dorsal root ganglion neurones. *Journal of Physiology* **359**, 431–466.

FERNANDEZ, J., FOX, A. P. & KRASNE, S. (1984). Membrane patches and whole-cell membranes: a comparison of electrical properties in rat clonal pituitary (GH3) cells. *Journal of Physiology* **356**, 565–585.

FISCHBACH, G. & SCHUETZE, S. (1980). A post-natal decrease in acetylcholine channel open time at rat end-plates. *Journal of Physiology* **303**, 125–137.

FRIEDMAN, M. E., SUAREZ-KURTZ, G., KACZOROWSKI, J., KATZ, G. M. & REUBEN, J. P. (1986). Two calcium currents in a smooth muscle cell line. *American Journal of Physiology* **250**, H699–703.

GONOI, T. & HILLE, B. (1987). Gating of Na channels: inactivation modifiers discriminate among models. *Journal of General Physiology* **89**, 253–274.

GONOI, T., SHERMAN, S. & CATTERALL, W. A. (1985). Voltage-clamp analysis of tetrodotoxin-sensitive and -insensitive sodium channels in rat muscle cells developing *in vitro*. *Journal of Neuroscience* **5**, 2559–2564.

HAGIWARA, S. (1983). *Membrane Potential-Dependent Ion Channels in Cell Membrane: Phylogenetic and Developmental Approaches*. New York: Raven Press.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HARRIS, J. B. & MARSHALL, M. W. (1973). Tetrodotoxin-resistant action potential in newborn rat muscle. *Nature* **243**, 191–192.

HILLE, B. (1984). *Ionic Channels of Excitable Membranes*. Sunderland, MA, U.S.A.: Sinauer Associates Inc.

KANO, M., WAKUTA, K. & SATOH, R. (1987). Calcium channel components of action potential in chick skeletal muscle cells developing in culture. *Developmental Brain Research* **32**, 233–240.

KNUDSON, C. M., JAY, S. D. & BEAM, K. G. (1986). Developmental increase in skeletal muscle slow calcium current. *Biophysical Journal* **49**, 13a.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1986). Evidence for two distinct calcium channels in rat vascular smooth muscle cells in short-term primary culture. *Pflügers Archiv* **407**, 566–568.

MATTESON, D. R. & ARMSTRONG, C. M. (1986). Properties of two types of calcium channels in clonal pituitary cells. *Journal of General Physiology* **87**, 161–182.

MILLER, J. R. (1987). Multiple calcium channels and neural function. *Science* **235**, 46–52.

NARAHASHI, T., TSUNOO, A. & YOSHII, M. (1987). Characterization of two types of calcium channels in mouse neuroblastoma cells. *Journal of Physiology* **383**, 231–249.

NILIUS, B., HESS, P., LANSMAN, J. B. & TSIEN, R. W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature* **316**, 443–446.

NOWYCKY, M. C., FOX, A. P. & TSIEN, R. W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440–443.

PURVES, D. & LICHTMAN, J. W. (1980). Elimination of synapses in the developing nervous system. *Science* **210**, 153–157.

REDFERN, P. A. (1970). Neuromuscular transmission in new-born rats. *Journal of Physiology* **209**, 701–709.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. II. The action of tetrodotoxin. *Acta physiologica scandinavica* **82**, 70–78.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569–574.

RUBIN, L. L. (1985). Increases in muscle Ca^{2+} mediate changes in acetylcholinesterase and acetylcholine receptors caused by muscle contraction. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 7121–7125.

SAKMANN, M. & BRENNER, H. R. (1978). Changes in synaptic channel gating during neuromuscular development. *Nature* **276**, 401–402.

SALMONS, S. & SRÉTER, F. A. (1976). Significance of impulse activity in the transformation of skeletal muscle type. *Nature* **263**, 30–34.

SÁNCHEZ, J. A. & STEFANI, E. (1978). Inward calcium current in twitch muscle fibres of the frog. *Journal of Physiology* **283**, 197–209.

SHERMAN, S. J. & CATTERALL, W. A. (1982). Biphasic regulation of development of the high-affinity saxitoxin receptor by innervation in rat skeletal muscle. *Journal of General Physiology* **80**, 753–768.

SHERMAN, S. J., CHRIVIA, J. & CATTERALL, W. A. (1985). Cyclic adenosine 3′:5′-monophosphate and cytosolic calcium exert opposing effects on biosynthesis of tetrodotoxin-sensitive sodium channels in rat muscle cells. *Journal of Neuroscience* **5**, 1570–1576.

STANFIELD, P. R. (1977). A calcium dependent inward current in frog skeletal muscle fibres. *Pflügers Archiv* **368**, 267–270.

STEINBACH, J. H., MERLIE, J., HEINEMANN, S. & BLOCH, R. (1979). Degradation of junctional and extrajunctional acetylcholine receptors by developing rat skeletal muscle. *Proceedings of the National Academy of Sciences of the U.S.A.* **76**, 3547–3551.

STUREK, M. & HERMSMEYER, K. (1986). Calcium and sodium channels in spontaneously contracting vascular muscle cells. *Science* **233**, 475–478.