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Acetylcholine Sensitivity and Distribution on Mouse Neuroblastoma Cells

Abstract. *Action potentials resulted from iontophoretic application of acetylcholine to cloned mouse neuroblastoma cells maintained in culture. Cell bodies were sensitive to acetylcholine, but half the cells were insensitive along one of their processes, suggesting an analogy with normal axons and dendrites. Some cells were electrically coupled via their processes, but no chemically mediated synaptic transmission was seen.*

Sensitivity of innervated skeletal muscle fibers to acetylcholine (ACh) is confined to regions of the cell surface close to the synaptic terminals at the end plate. After denervation this sensitivity increases and covers the whole muscle fiber surface (*1*). Innervated parasympathetic neurons are most sensitive to ACh close to synaptic endings but develop a uniform high sensitivity after degeneration of the presynaptic nerve fibers (*2*). Embryonic muscle, like denervated muscle, is receptive to innervation and is sensitive to ACh over its whole surface (*3*), but it is not known whether the same is true of newly differentiated nerve cells before they receive synaptic contacts.

To study the sensitivity of newly formed nerve cells to transmitter, we have used a cloned line of mouse neuroblastoma cells (*4*). This line is capable of morphological "differentiation" in vitro, and thus provides a simple system for studying this question. These cells are propagated in suspension as round cells, but if they are plated on a surface of glass, collagen, or acid-treated plastic, many send out processes and take on some of the morphology of nerve cells. The plated cells show considerable variations in form, ranging from round cells that are distinguishable from the suspended cells only by their larger size, through cells with as many as a dozen short processes, to cells with one or two processes that are several millimeters in length.

Physiological properties of cells plated in plastic tissue-culture dishes and cultured for from 3 to 5 weeks were investigated by intracellular re-

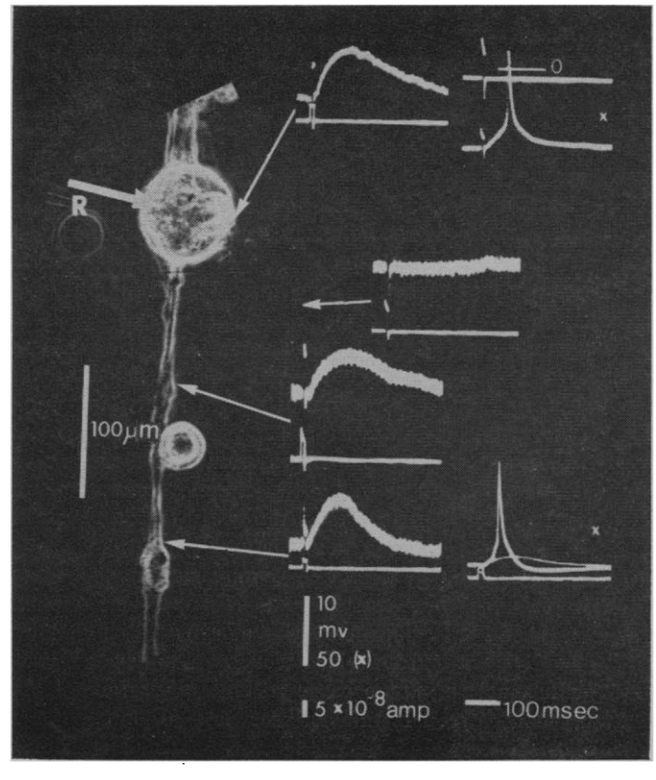

Fig. 1. Distribution of sensitivity to acetylcholine on the soma and process of a neuroblastoma cell 1 month after plating. The top of each pair of traces shows the potential change elicited by iontophoretic application of ACh to the point on the cell surface marked by the corresponding arrow. The current through the ACh electrode is monitored in each bottom trace (10-msec pulses were used in these and all subsequent records). The position of the recording electrode is marked by the heavy arrow (*R*). The top two sets of records show the response to ACh applied to the cell body; the left-hand set, at high gain (calibration, 10 mv), shows an ACh potential (responses of similar magnitudes and time course resulted no matter to which point on the soma the ACh electrode was applied); the right-hand set, at lower gain (calibration, 50 mv), shows the response to a larger pulse of ACh (the current trace is lifted; *O* marks the line of zero resting potential) where an action potential was triggered by the ACh potential. The sets of traces below show responses to ACh applied to the cell process, and as a control for diffusion of ACh, the response to ACh delivered into the bath. The set of records at bottom right, at lower gain, shows superimposed traces of a subthreshold ACh potential and a potential large enough to trigger a spike in the cell process. The 100-msec time scale applies to all records. Records at lower gain (calibration, 50 mv) are marked x.

cording, with the use of a bridge circuit for intracellular application of current and a second micropipette for iontophoretic application of ACh or for passing current into neighboring cells (*5*).

Usually, cells with a nerve-like appearance (*6*) were selected for investigation. Resting potentials were mostly about 40 mv immediately after penetration and increased to 50 to 60 mv within the next few minutes. Probably the true resting potentials were always greater than those seen. All cells showed some degree of electrogenic response to current applied through the bridge circuit. This finding differs from that of Nelson *et al.* (*7*) who found that some of the cells in an uncloned neuroblastoma culture gave no electrogenic response. The variable morphology of the cells, even within the cloned line we used, suggests that caution must be used in interpreting physiological differences between cells to be stages in a sequence of development. Action potentials, whether elicited by current from the bridge or by iontophoretic ACh, were usually similar to those seen in recordings from sympathetic nerve cells in vivo (*8*) (Fig. 1), but occasional strikingly different forms were seen. For example, in some cells the spike was followed by a prolonged plateau of depolarization like the action potential of heart muscle cells. Propagated impulses could be initiated in the processes as well as in the cell bodies. This is illustrated in Fig. 1 where ACh, released by a 10-msec current pulse, was applied to a process at a point 200 μm from the cell body, and an action potential was set up.

All cells investigated, whether or not they had processes, could be depolarized by extracellular application of ACh. No recordings were made from the small round cells from suspension culture. The surface of a cell body was uniformly sensitive to ACh. Many spots were tried on the surface of individual cells, and all gave responses similar in both amplitude and waveform; none showed a fast rise after a delay, which would have been indicative of localized spots of high sensitivity. The absolute degree of ACh sensitivity was lower (by one to two orders of magnitude) than that of frog parasympathetic ganglion cells, the only vertebrate nerve cells for which this parameter is known (*2*).

Although cell processes sometimes made contacts with other processes or cell bodies, no evidence of chemically mediated synaptic transmission was seen. A number of cells, however, were coupled by low-resistance, nonrectifying, electrical junctions between their processes or between processes and cell bodies. Lowenstein and Kanno (*9*) suggest that an inability to form cell contacts is a causative factor in carcinogenesis. Our finding agrees with the results of Furshpan and Potter (*10*) who found that cultures of malignant cells can form low-resistance junctions.

Not all cells were sensitive to ACh along the length of their processes. Of the nine cells in which this point was investigated five had a process which was relatively insensitive to ACh from

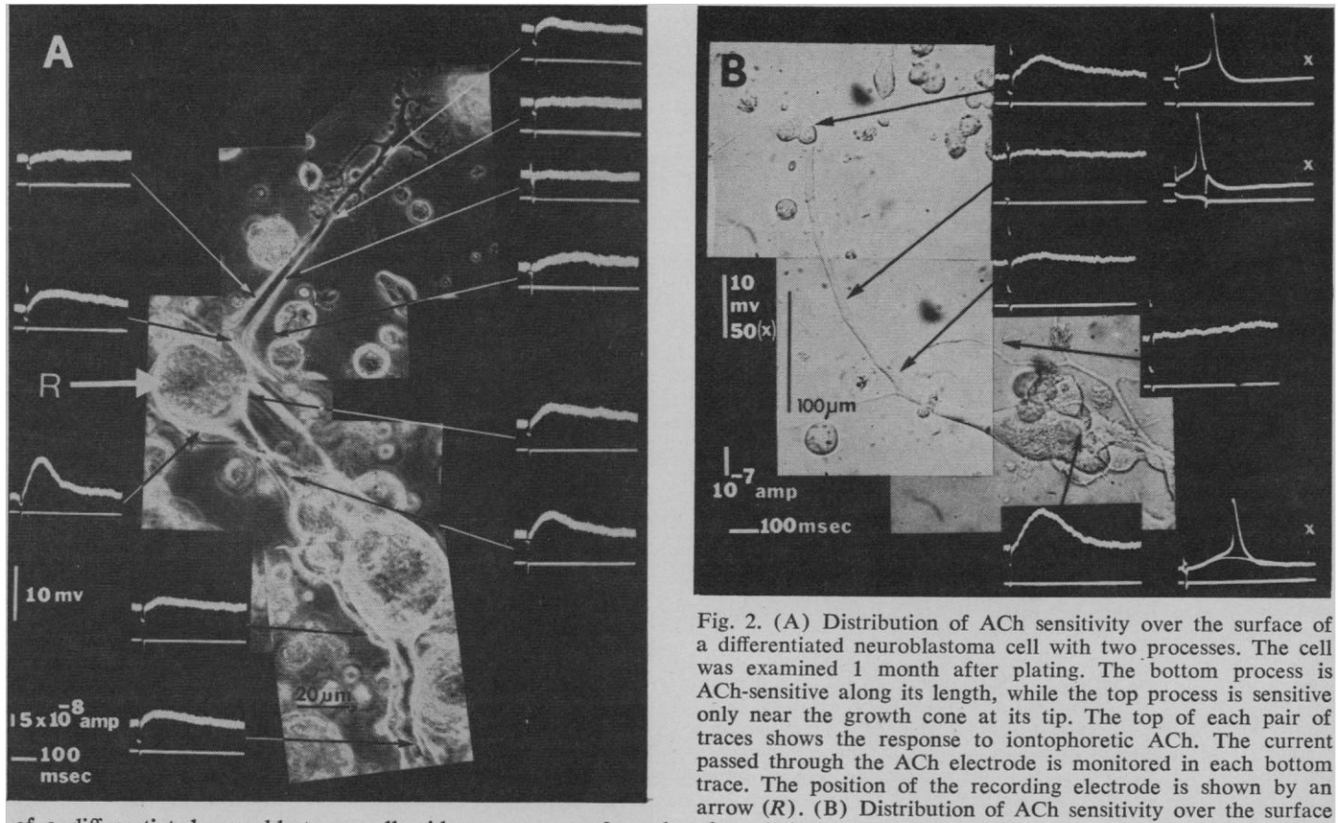

Fig. 2. (A) Distribution of ACh sensitivity over the surface of a differentiated neuroblastoma cell with one process, 3 weeks after plating. Sensitivity to ACh is restricted to the cell body, where ACh application evoked an action potential (lower right) and to the axon tip. Extracellular electrical stimulation of the axon or its tip gave rise to an action potential which propagated back to the cell body (two top right traces; points of stimulation as for ACh application). (B) Distribution of ACh sensitivity over the surface of a differentiated neuroblastoma cell with two processes. The cell was examined 1 month after plating. The bottom process is ACh-sensitive along its length, while the top process is sensitive only near the growth cone at its tip. The top of each pair of traces shows the response to iontophoretic ACh. The current passed through the ACh electrode is monitored in each bottom trace. The position of the recording electrode is shown by an arrow (*R*).

close to the cell body to near its growing tip. The region near the tip was always sensitive, but the response here differed from that of the cell body in that it was very easily desensitized by repeated pulses of ACh. Several minutes were required between pulses in order to restore maximum sensitivity (Fig. 2).

Figure 2A shows a cell with two processes, one of which was sensitive only near its tip, which had the appearance of a typical growth cone. The other process followed a tortuous path past a number of other cells (no electrical coupling was found in this instance), ending near the bottom of the photograph, and was sensitive to ACh along its length. The cell in Fig. 2B had only one process, and this was insensitive to ACh except near its tip. Insensitive regions of membrane were still able to conduct action potentials, as shown in Fig. 2B, where extracellular electrical stimulation initiated action potentials both at the axon tip and along its length.

The growth cone at the tip of a process is where new membrane is laid down. The fact that some cell processes are sensitive to ACh only at their growing tips is interesting because it demonstrates that the sensitivity somehow becomes reduced proximal to the region of membrane growth.

Parasympathetic nerve cells in adult animals (*2*) are sensitive to ACh only at spots on the cell body and not along the axons. Similarly, in adult skeletal muscles, ACh sensitivity is localized to small spots. This localization takes place during the course of development but only after the muscle fiber receives a synapse. Cutting the nerve to an adult muscle causes reversion of the pattern of distribution of ACh sensitivity to the embryonic state, an indication that the restriction of ACh sensitivity to an end plate is dependent on the nerve (*1*). Furthermore, it has been suggested that the change in the distribution of ACh sensitivity which occurs when a synapse is formed is associated with a reduction in the receptivity of the cell to further innervation (*11*).

It has yet to be demonstrated that the physiology of tumor cells propagated in culture is the same as that of nerve cells in an animal. If it is, our results suggest that the pattern of distribution of ACh sensitivity on the surface of developing nerve cells may change before synaptic connections are formed, and thereby play a part in determining which regions of a cell receive synapses. The processes of the tumor cells sensitive and insensitive to ACh could be analogous to dendrites and axons, respectively. The loss of ACh sensitivity by a growing cell process (axon) might be associated with a change in the properties of its membrane which renders it incapable of receiving synaptic connections. On the other hand, processes which retain their chemosensitivity (dendrites) would remain capable of receiving synaptic contacts.

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Release of Protoplasts in the Yeast Phase of Histoplasma capsulatum without Added Enzyme

Abstract. *Cells of the yeast phase of the dimorphic systemic fungus pathogen* Histoplasma capsulatum *readily released large numbers of intact protoplasts without degradation of their cell walls by snail or microbial enzymes, previously regarded as a requirement for all yeast and mycelial fungal forms. Over 90 percent of "B" type yeast cells in the early logarithmic phase of growth released living protoplasts when incubated at 37°C with 2 molar magnesium sulfate, whereas "A" type yeast cells required prior exposure for 24 hours to 2-deoxy-*D*-glucose before incubation in the 2 molar magnesium sulfate.*

Histoplasma capsulatum exists as two morphological types (*1*) in the saprophytic multinucleate heterokaryotic mycelial phase: the "A" or albino type and the "B" or brown type. The parasitic or yeast phase uninucleate forms of these two types are indistinguishable morphologically by phase microscopy or staining reactions, and they cannot be differentiated by physiological tests. We succeeded in releasing living protoplasts from both the "A" and "B" mycelial forms of *H. capsulatum* by means of wall degradation by snail gastric enzymes (*2, 3*). However, we were unable to induce significant protoplast formation from the yeast forms of the same organism by this procedure. In order to study indirectly possible differences in cell wall structure and composition, physiology, and cytology of the "A" and "B" type yeast phases, we sought to induce the release of their protoplasts in an osmotically stable and chemically defined medium.

Magnesium sulfate, at concentrations ranging from 0.8 to 1*M*, is the most consistent osmotic stabilizer of yeast protoplasts and is also an inhibitor of bacterial contamination when used in conjunction with enzyme treatment (*3*). For this reason, we tested the effect of $MgSO_4$ alone at molar concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 on the growth and morphological stability of intact *H. capsulatum* yeast cells. No enzyme or other wall-softening agents were added.

When the G-184B and G-186B yeast cultures in 2*M* $MgSO_4$ broth (*4*) were examined after 48 hours of incubation at 37°C, we noticed that large numbers of protoplasts were being released. After the cultures were incubated for 96 hours, they contained