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INFLUENCES ON THE EXPRESSION OF ACETYLCHOLINE RECEPTORS ON RAT NODOSE NEURONES IN CELL CULTURE

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SUMMARY

1. Nodose neurones dissociated from new-born rats were grown in culture in the absence or presence of cells from neonatal skeletal muscle or heart.

2. In cultures devoid of non-neuronal cells cholinergic interactions between the neurones were common. In the presence of non-neuronal cells such interactions were rare.

3. A decrease in the proportion of neurones responsive to ACh was primarily responsible for the reduced incidence of synaptic interactions. Non-neuronal cells influenced the expression of ACh receptors in developing nodose neurones in culture.

4. Most neurones appeared susceptible to the non-neuronal influence during the first week in culture.

5. Many nodose ganglion neurones, whether grown in the presence or absence of non-neuronal cells, were sensitive to γ-aminobutyric acid and serotonin but were insensitive to glutamate, glycine and L-epinephrine.

INTRODUCTION

In the preceding paper we reported the presence of excitatory synapses between dissociated neurones from the nodose ganglia of new-born rats when these neurones were grown in low-density cell culture in the near absence of non-neuronal cells (Baccaglini & Cooper, 1981). Transmission at these synapses was blocked by conventional ganglionic nicotinic antagonists, suggesting that transmission is primarily cholinergic and mediated by ganglionic acetylcholine (ACh) receptors. In this paper we describe environmental conditions which appear to influence the development of these synapses. When the neurones were grown in the presence of non-neuronal cells (skeletal myotubes or myocytes) the incidence of synaptic interactions was markedly reduced. This effect was mainly due to a decrease in the proportion of neurones sensitive to ACh, indicating that these non-neuronal cells can influence the presence of ACh receptors on developing nodose neurones in cell culture.

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METHODS

Cell preparations, media and growth conditions for nodose ganglion neurones were described in the preceding paper (Baccaglini & Cooper, 1981).

'Nerve–muscle' cultures. In this series of experiments sister cultures were grown by themselves or in the presence of non-neuronal cells dissociated from heart or pectoral muscles. The heart or, more frequently, skeletal muscles of new-born rats were enzymically dissociated with collagenase (Worthington, type I, 1 mg/ml.); the digestion took place at 37 °C for three successive periods (20 min each). The supernatants of the last two periods were collected and washed three times with 'growth' medium. In some cases, the cell suspension was first filtered (10–20 μm millipore filter) to remove small clumps of cells and then pre-plated in a Falcon culture flask for 20 min at 37 °C to remove more rapidly adhering cells (e.g. fibroblasts). The supernatant, now enriched in muscle cells, was then collected, counted and plated. Cell division was subsequently arrested by irradiation from a ^{60}Co source when the cells had formed a monolayer. By this time several large myotubes had formed in the cultures of cells from skeletal muscle. In some cases ('neurone–muscle' cultures) the neurones were plated onto a pre-formed monolayer of muscle cells. In other cases twenty-five thousand myoblasts were either plated at the time the neurones were plated or 1 week later. The myoblasts were then allowed to divide until they had formed a monolayer (usually 3–4 days). Cell division was arrested by irradiation as described above. Often it was difficult to keep viable myotubes for longer than 3–4 weeks in culture.

ACh sensitivity was tested by means of a pressure ejection system. Micropipettes with broken tips (5–40 μm diameter) were filled with ACh solutions (10^{-7}–10^{-3} M) and connected to a tank of compressed air. Brief pressure pulses, gated by a solenoid valve, caused ejection of 'puffs' of solution (Choi & Fischbach, 1981). The pipette could be positioned under visual control and chemicals applied to a restricted region of the neurone quickly and reproducibly. The area affected by the puff could be varied from a few tens to hundreds of microns, depending on the pressure applied to the pipette and diameter of its tip. With this method, in contrast to the ionophoretic technique, the maximum local concentration of the applied substance is known, although the actual concentration at the cell body is presumably lower. Some dilution of the drug occurred, especially with larger tip pipettes (40 μm) by backflow from the bath into the pipette. In some experiments (the dose–response curves) this dilution was reduced by applying a small 'bias' pressure to the drug pipette. The amount of 'bias' pressure was adjusted by suspending small oil drops in the drug solution, and observing their flow from the tip of the pipette.

To measure the percentage of neurones in one culture sensitive to ACh, we usually recorded intracellularly from fifty neurones chosen at random and observed changes in membrane potential when an ACh (10^{-3} M) solution was pressure-ejected onto the neurone's cell body; only neurones with resting potentials of at least -35 mV were tested. A neurone was scored as sensitive to ACh if ACh produced a depolarization of at least 5 mV, although most of the sensitive neurones were depolarized by 15–30 mV. A neurone which displayed no detectable change in membrane potential was scored as insensitive. Neurones which depolarized by 2–3 mV were rarely seen and were not scored. The electrophysiological techniques and perfusion solution were described in the preceding paper (Baccaglini & Cooper, 1981).

Pressure ejection was also used to test the sensitivity of the neurones to several other substances: γ-aminobutyric acid (GABA; 10^{-3} M); serotonin (5-HT; 10^{-4}–10^{-3} M); glutamate (10^{-4}–10^{-3} M); glycine (10^{-3} M); L-epinephrine (10^{-4}–10^{-3} M). All agents were dissolved in perfusion solution.

Pharmacological block of exogeneously applied ACh. Pipettes of similar size (10–30 μm) were filled with a solution containing ACh (3×10^{-5} M), or ACh (3×10^{-5} M) and hexamethonium (10^{-4} M), or ACh (3×10^{-5} M) and atropine (10^{-6} M). First the response of a sensitive neurone to 'puff' of ACh solution was tested (three to four times) in normal solution with the pipette tip at a standard distance from the neurone (usually 40–50 μm). A solution containing hexamethonium (10^{-4} M) was then introduced through the perfusion system for sufficient time (*ca.* 10 min) to allow complete replacement of the fluid surrounding the cells. The ACh pipette was replaced with one filled with both ACh and hexamethonium and puffs of this solution were applied (three to four times). In this way, puffs of ACh could be tested without causing local changes in hexamethonium concentration. Responses to puffs of ACh in normal solution were then re-assayed. The effect of atropine on the ACh response was investigated in the same way.

Dose–response relationship. Pipettes with uniform tip diameter (*ca.* 40 μm) were filled each with

an ACh solution of different concentration (10^{-7}–10^{-3} M). Each pipette in turn was positioned at a standard location with respect to the neurone (40–50 μm) and the effects of three to four puffs were observed. Such tests of different concentrations of ACh were made at intervals of about 5 min.

Conditioned medium. Conditioned medium was obtained in a similar fashion to that described by Patterson & Chun (1977). Briefly, heart or pectoral muscles from three to five animals were dissociated by enzymic digestion as previously described. The cell suspension was then plated into Falcon culture flasks (75 cm). The cells were left to proliferate in 20–30 ml. 'growth' medium to which no nerve growth factor (NGF) or methocel had been added. The supernatant was collected every other day and stored frozen. This conditioned medium was thawed and mixed with an equal volume of fresh growth medium; the concentrations of NGF and methocel were adjusted to the final concentration as described before (Baccaglini & Cooper, 1981).

Data analysis. The 'error' bars of the histograms represent the 99·5 % confidence level that the experimental data represent the true population, as calculated by means of the binomial distribution

$$p(x) = \frac{N!}{x!\,(n-x)}!\,(p)^x\,(q)^{N-x}$$

where N is the total number of neurones, x is the number of cells experimentally found to be sensitive to ACh, p is the probability of finding a cell sensitive to ACh and q is $1-p$.

RESULTS

Growth and electrophysiological properties of nodose ganglion neurones co-cultured with muscle cells

The growth characteristics of nodose neurones grown in the presence of skeletal myotubes, fibroblasts and cardiocytes were essentially similar to those described in the previous paper for nodose neurones grown alone. Within 24 hr of plating, the neurones attached to the non-neuronal cells and started to grow processes. By 3–4 weeks the neuronal somata had increased to 30–40 μm in diameter. The neuronal processes formed bundles lying above or below the muscle cells; in regions of high density of non-neuronal cells the processes were often obscured and the neuronal cell bodies were embedded. The number of neurones remained approximately the same during the 4–6 week period studied, indicating that very little neuronal cell death occurred (less than 10 % in most cultures). The resting potentials, the amplitudes and shapes of the action potentials, and the sensitivities of the action potentials to tetrodotoxin (TTX), Na^+ replacement and Co^{2+} of many nodose neurones co-cultured with muscle were similar to those described in the preceding paper for nodose neurones cultured in the virtual absence of non-neuronal cells (Baccaglini & Cooper, 1981).

Synaptic interactions

A striking difference between sister cultures of nodose neurones grown in the virtual absence of non-neuronal cells (neurone-alone cultures) and those grown in the presence of muscle cells (neurone–muscle cultures) was in the incidence of synaptic interactions. In the 'neurone–muscle' cultures we were often unable to detect any spontaneous synaptic activity. The largest percentage of neurones exhibiting spontaneous synaptic activity (10 %), recorded in a few neurone–muscle cultures, was well below the value typical for neurone-alone cultures (50 %). In addition, in less than 3 % of over 235 randomly sampled pairs did one neurone evoke an excitatory post-synaptic potential (e.p.s.p.) on the other. In these relatively rare cases cholinergic

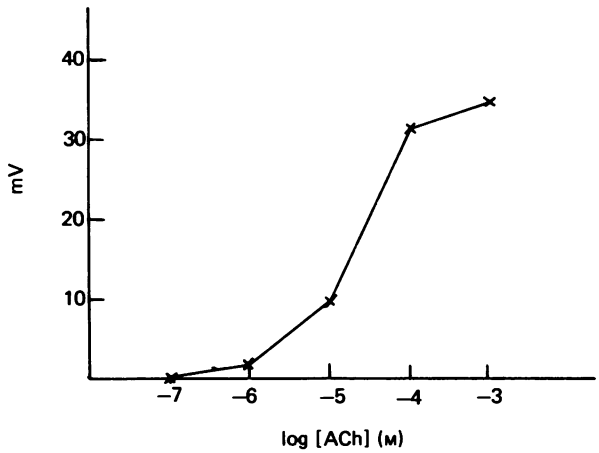

Fig. 1. Responses of a single neurone to five concentrations of ACh. Several pipettes each filled with a different concentration of ACh (10^{-7}–10^{-3} M) were successively positioned at the same distance from the neuronal soma and the responses to standard 'puffs' of ACh solution recorded. The amplitude of the depolarization produced by each concentration of ACh was plotted against the logarithm of the concentration.

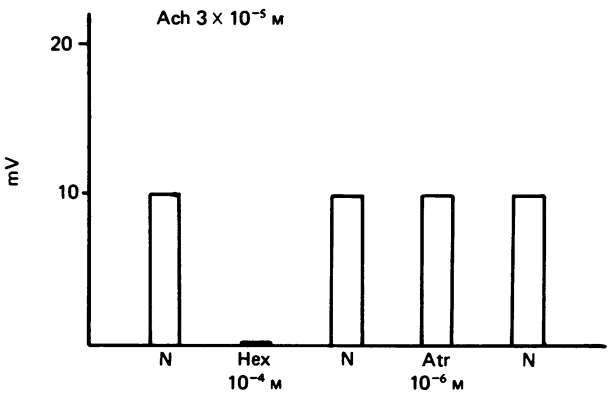

Fig. 2. Pharmacological block of the response to exogenously-applied ACh. The neuronal response to four standard 'puffs' of 3×10^{-5} M-ACh solution was first recorded in normal perfusion solution (N). The neuronal response to four 'puffs' of 3×10^{-5} M-ACh plus 10^{-4} M-hexamethonium was then recorded in perfusion solution containing 10^{-4} M-hexamethonium (Hex). The response to the ACh solution in normal medium was re-assayed before repeating the procedure with 10^{-6} M-atropine (Atr) in place of hexamethonium. The size of the depolarization in each solution is shown on the vertical axis.

antagonists (e.g. hexamethonium) reversibly blocked the e.p.s.p.s. In addition, although we did not record from myotubes routinely in this study, we did observe spontaneous excitatory junctional potentials (e.j.p.s) from some myotubes, and neuronally evoked e.j.p.s were detected in the small number of neurone-myotube pairs examined. Therefore the ability of some neurones to synthesize and release ACh was also present in co-culture with muscle, suggesting that an alteration in presynaptic mechanisms (e.g. synthesis or release of ACh) could not easily explain the decrease in the number of synaptic interactions among neurones in the neurone–muscle cultures. An alternative explanation for this decrease is an alteration of post-synaptic mechanisms (e.g. density or effectiveness of ACh receptors).

ACh sensitivity

Neuronal sensitivity to ACh was tested by means of brief pressure-induced ejections of a solution of ACh onto the neuronal somata (see Methods). Before testing a large number of neurones, we determined the dose–response relationship of five ACh-sensitive nodose neurones. A typical dose–response curve is shown in Fig. 1. The half-maximal response was elicited by the pipette containing 3×10^{-5} M-ACh; maximal or nearly maximal responses were produced by 10^{-3} M-ACh. The depolarization evoked by a puff of 3×10^{-5} M-ACh was reversibly blocked by adding nicotinic antagonists such as 10^{-4} M-hexamethonium to the perfusion medium but was unaffected by 10^{-6} M-atropine (Fig. 2). These results suggest that the receptors which responded to exogenously applied ACh were similar to those which mediated the synaptic interactions (see Baccaglini & Cooper, 1981).

A 10^{-3} M-ACh solution was used routinely to test neuronal sensitivity. With this concentration we found that the neurones fell into two categories: 'sensitive' and 'insensitive'. A typical depolarization produced by a puff of 10^{-3} M-ACh onto a sensitive neurone is shown in Fig. 3*A* and an example of an insensitive neurone is shown in Fig. 3*B*. Most sensitive neurones (90%) depolarized 20–35 mV (Fig. 3*A*) to a puff of ACh solution; the remaining 10% depolarized only 5–10 mV.

Influence of non-neuronal cells on ACh sensitivity. Sister neuronal cultures were grown either in the absence or in the presence of muscle cells from the time of plating. 500–600 neurones were plated per culture dish from which fifty were chosen at random to determine the proportion of ACh-sensitive neurones in that dish. These assays were made at two different times: 1½–2 weeks and 3–4 weeks *in vitro*. The results of three such experiments are shown in Fig. 4. Neurone–muscle cultures had significantly fewer ACh-sensitive neurones than their sister cultures grown without non-neuronal cells at 10–14 days in culture; the proportion of ACh-sensitive neurones did not change for either conditions for at least another 2 weeks. There was quantitative variability from plating to plating: in Fig. 4*B* 15–30% of the neurones in the neurone–muscle cultures were sensitive to ACh at 10–14 days, whereas in Fig. 4*A* 40% of the neurones in the neurone–muscle cultures were sensitive to ACh at this time. Forty percent represents the highest proportion of ACh sensitive neurones in neurone–muscle cultures and was infrequently observed (two of ten platings); more typical values were 0–30%. The platings shown in Fig. 4*C* had a higher proportion (80–100%) of ACh-sensitive neurones in the neurone-alone cultures than those in Fig. 4*A* and *B*. In this plating two variables were changed from our standard culture condition; one

was that methocel was omitted from the growth medium, and the other was that the cultures were fed every 2 days instead of every 4 days. In two other platings in which the neurones were grown under similar conditions we consistently found 70–100% of the neurones sensitive to ACh.

The possibility existed from these results that some non-neuronal factor(s), either added to the medium or depleted from it, could influence the nodose neurones' sensitivity to ACh. In a few platings we tested whether medium conditioned by muscle

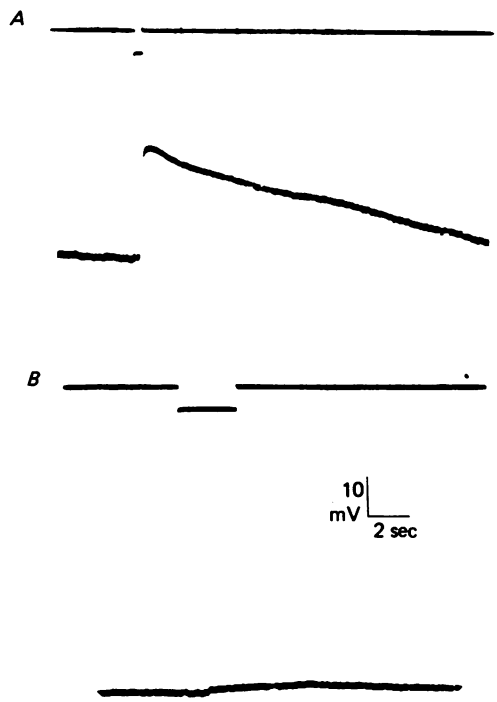

Fig. 3. Tests of sensitivity to ACh. An ACh solution was pressure ejected onto the soma of the neurone from a 'puffer' pipette (tip diameter about 40 μm), while recording the membrane potential with an intracellular micro-electrode. *A*, a neurone sensitive to ACh: a puff (duration 0·4 sec) of ACh solution (10^{-3} M) caused a rapid depolarization (26 mV), followed by re-polarization as ACh diffused away from the neurone. Resting potential −50 mV. *B*, a neurone insensitive to ACh: a similar puff of the same ACh solution had no detectable effect on a nearby neurone. The small drift of the base line was caused by a flow artifact which persisted when the micro-electrode was withdrawn from the cell. Resting potential −55 mV.

cells could convey this influence. We found no consistent differences in the proportion of neurones sensitive to ACh between neurone-alone cultures grown in control growth medium and neurone-alone cultures grown in 50% conditioned medium.

Temporal responsiveness of neurones to non-neuronal cell influences. To investigate the temporal responsiveness of neurones to non-neuronal cell influences, we grew neuronal sister cultures in three different conditions: in one group the neurones were grown in the virtual absence of non-neuronal cells; in a second group the neurones

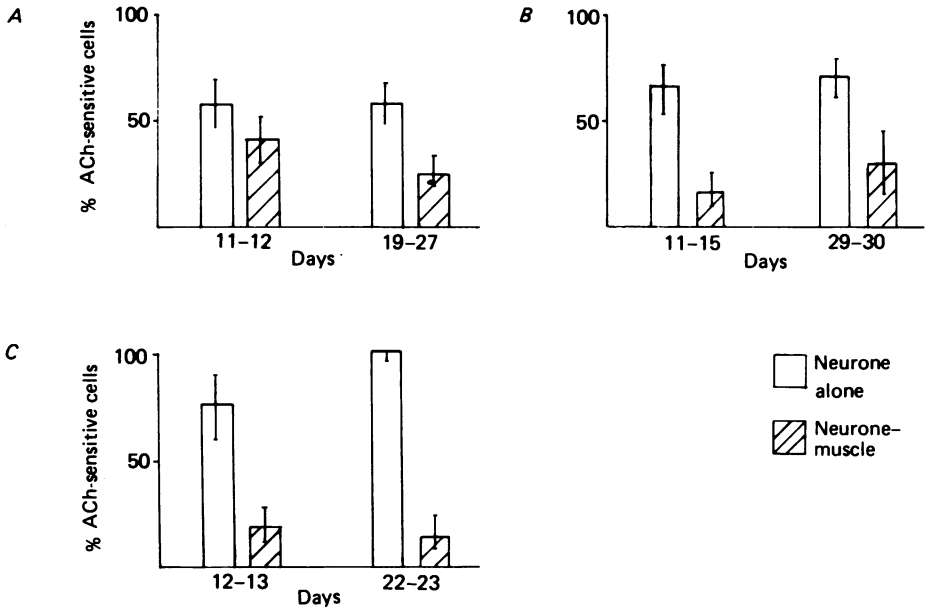

Fig. 4. Incidence of ACh-sensitive neurones grown in the presence or absence of non-neuronal cells in three different experiments (*A*, *B* and *C*). The percentage of sensitive neurones (vertical axis) was tested at two times after plating the neurones (horizontal axis). The data for each column were taken from at least 100 neurones (two to three dishes). Unfilled columns: tests on neurones in neurone-alone cultures; hatched columns: tests on neurones in the neurone–muscle cultures. The 'error' bars represent the 99·5 % confidence level (see text).

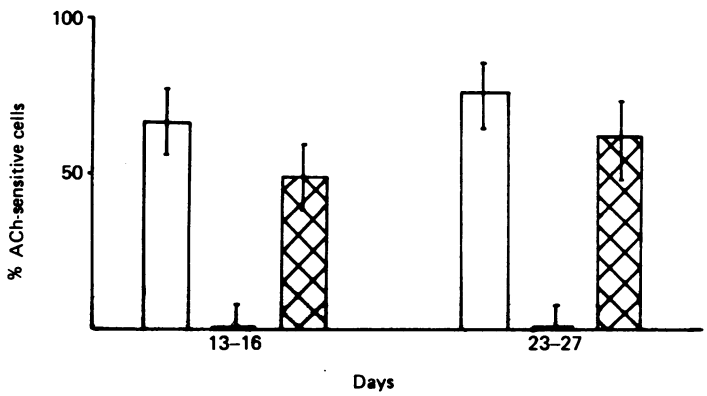

Fig. 5. The effect of the time of addition of non-neuronal cells on the incidence of ACh sensitivity. Twenty-five thousand muscle cells were added to sister cultures at the same time the neurones were plated (filled columns) and 1 week later (cross-hatched columns). Unfilled columns represents proportion of neurones sensitive to ACh in cultures from the same plating grown in the absence of non-neuronal cell. ACh sensitivity was assayed at times shown on the horizontal axis. The 'error' bars represent the 99·5 % confidence level (see Methods).

were co-cultured with skeletal myotubes, and in a third group the neurones were grown initially in the virtual absence of non-neuronal cells, then after one week individual myoblasts were added to these cultures; these myoblasts continued to divide for about 3 days to form a monolayer of myocytes and myotubes. Each group was then examined for the proportion of ACh-sensitive neurones both at 10–14 days and at 25–30 days after plating the neurones. The result of one such plating is shown in Fig. 5.

In the virtual absence of non-neuronal cells 70 % of the neurones were sensitive to ACh. No ACh-sensitive neurones were detected in those cultures in which neurones

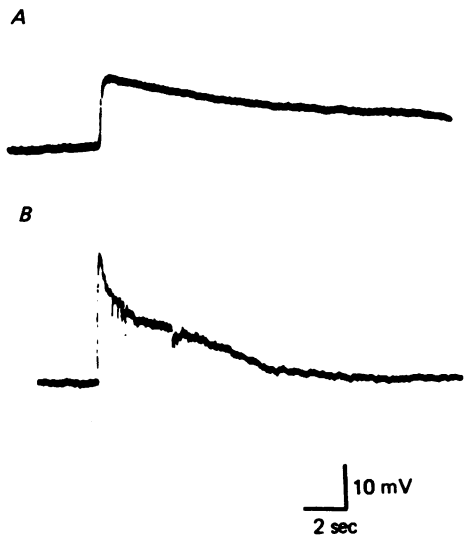

Fig. 6. Responses of nodose neurones to GABA and 5-HT. *A*, this neurone was depolarized (12 mV) by GABA (10^{-3} M) puffed onto its soma (neurone–muscle culture). Resting potential −50 mV. *B*, this neurone was depolarized (26 mV) by 5-HT (5×10^{-4} M) puffed onto its soma (neurone-alone culture). The neurone fired several action potentials (only the undershoot is visible) during the repolarizing phase. Resting potential −68 mV. The arrows indicate the beginning and the end of the 'puff'.

were plated at the same time as the muscle (second group). However in cultures where the neurones were grown initially without non-neuronal cells, and the myoblasts added 1 week later, 50 % of the neurones were sensitive to ACh. In another experiment myoblasts were added only 2–3 days after plating the neurones. In this case, only 25 % of the neurones were sensitive to ACh compared to 85 % for sister cultures grown in the absence of muscle. These results indicate that ACh-sensitive nodose neurones could develop in the same culture as muscle; that is, muscle *per se* was not selectively destroying ACh-sensitive neurones. In addition these results suggest that there is a critical period, the first week in culture, during which neurones can respond to non-neuronal cell influences.

Sensitivity to other agents. Gamma-aminobutyric acid (10^{-3} M), 5-HT (10^{-4}–10^{-3} M), glycine (10^{-3} M) glutamate (10^{-4}–10^{-3} M), L-epinephrine (10^{-4}–10^{-3} M) were applied by pressure ejection to the somata of nodose neurones while simultaneously recording

the membrane potential. Only GABA and 5-HT caused a detectable change in membrane potential (depolarization in both cases) and only in some neurones (Fig. 6). Responses to both agents showed desensitization to a second puff of the test solution presented within 2 min of the first. Sixteen of forty-five neurones responded to GABA (depolarization 2–18 mV) and thirty-five of seventy-nine gave responses to 5-HT (depolarization 2–26 mV). Neurones sensitive to GABA and/or 5-HT were seen in both neurone-alone and neurone–muscle cultures. In addition both ACh-sensitive and insensitive neurones responded to GABA and/or 5-HT.

DISCUSSION

We found a significant reduction in the proportion of ACh-sensitive neurones in cultures where the neurones grew together with muscle, compared to cultures where the neurones grew in the virtual absence of non-neuronal cells. It seems unlikely that these differences can be explained by a selection of two distinct neuronal populations: one preferentially attaching and growing with muscle cells, and the other surviving in their absence. Non-neuronal cells were added as late as 2–3 days after plating the neurones, by which time many neurones had attached and sent out processes; however the proportion of ACh sensitive neurones 10 days later was significantly reduced compared to cultures which did not receive muscle; neuronal numbers during this time remained approximately the same. Further evidence against an adverse effect of muscle on ACh-sensitive neurones comes from experiments in which muscle was added 1 week after the neurones had been plated. In these cultures as many as 60 % of the neurones were sensitive to ACh even though they had been co-cultured with muscle for 3–4 weeks. These latter experiments also suggest that the first week in culture is a crucial period during which the neurones are receptive to the non-neuronal cell influence.

There is no information on the proportion of ACh-sensitive neurones in the new-born nodose ganglion of the rat. Consequently we do not know if neurones start off insensitive to ACh and only those cultured in the virtual absence of non-neuronal cells develop ACh sensitivity, or whether most neurones are ACh-sensitive to begin with and lose their sensitivity when co-cultured with muscle. The earliest that we were able to examine neurones electrophysiologically was after 10 days in culture, and subsequently the proportion of ACh-sensitive neurones stayed roughly constant for at least 3–4 more weeks. Almost all (90 %) neurones tested fell sharply into the categories of ACh-sensitive (with depolarizations of 15–35 mV) or ACh-insensitive (no apparent depolarization). Only 10 % gave small depolarizations (5–10 mV) to the standard test, possibly representing a distinct group of neurones in an intermediate state of acquisition or loss of ACh sensitivity. We tested whether the non-neuronal cells exerted their influence via the culture medium by growing neurones in medium which had been conditioned by muscle cells, however we found no effect on the proportion of ACh-sensitive neurones. Conceivably the non-neuronal cell influence is mediated by direct contact between neurones and muscle. If so, it may be possible to investigate contact-mediated effects by growing neurones on killed non-neuronal cells (Hawrot, 1980).

Only ACh sensitivity seemed to be affected by the presence of non-neuronal cells.

In fact, sensitivity to GABA and 5-HT, two other compounds known to excite many nodose neurones *in vivo* (De Groat, 1972; Sampson & Jaffe, 1974; Higashi, 1977) seemed similar for neurones grown in the virtual absence or in the presence of non-neuronal cells. The significance of ACh receptors on nodose neurones is unclear. Some nodose somata (Higashi, 1980), unmyelinated nodose axons (Armett & Ritchie, 1961) and vagal sensory afferents (Diamond, 1955) have been shown to be sensitive to ACh.

The reduction in the number of neurones sensitive to ACh when co-cultured with muscle may account in part or entirely for the reduced incidence of cholinergic synapses between neurones. If so, then one might expect to find abundant synaptic interaction in those cultures in which the neurones were grown alone initially and muscles added after 1 week, since the majority of neurones were sensitive to ACh in this condition. Experiments to test this point are in progress. Furthermore, we do not know if morphological but electrically silent neurone–neurone synapses form between neurones which lack ACh receptors; electrically silent synapses have been shown to form between adrenergic sympathetic neurones in culture (Landis, 1976). *In vivo* adult nodose neurones, like other sensory neurones, make synapses in the central nervous system but do not receive synapses on their somata or peripheral processes (reviewed by Lieberman, 1976). It is not known how this adult situation, characteristic of primary sensory neurones, is achieved during development. The cultures, in which the cellular and fluid environments of the neurones can be manipulated, may supply clues to such questions.

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