SHORT COMMUNICATIONS

CONDITIONING FACTOR(S) PRODUCED BY SEVERAL MOLLUSCAN SPECIES PROMOTE NEURITE OUTGROWTH IN CELL CULTURE

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The ability to correlate the behaviour of neurones in intact ganglia with events in isolation as has been shown in the leech (Ready & Nicholls, 1979; Fuchs, Nicholls & Ready, 1981), may provide insight into the intrinsic properties of individual neurones.

The culture of molluscan neurones is relatively new and the exact procedures for the control of environmental conditions necessary for obtaining neurite outgrowth have not been uniformly established. In early experiments, Chen, von Baumgarten & Takeda (1971) isolated viable identified Aplysia neurones, but were not able to obtain neurite outgrowth. Geletyuk (1977), using dissociated neurones from Lymnaea, demonstrated for the first time the regeneration of neurites in cell culture. Outgrowth has been reported by Kaczmarek, Finbow, Revel & Strumwasser (1979) and Dagan & Levitan (1981) for neurones isolated from several ganglia in Aplysia.

Recently we reported (Wong, Hadley, Kater & Hauser, 1981) that isolated neurones from adult central ganglia of Helisoma required the presence of co-cultured, intact Helisoma brains or brain-conditioned medium in order for significant neuritic outgrowth to occur. The need for a growth-promoting factor was also suggested by Proshansky, Schacher & Camardo (1981), who found that addition of Aplysia haemolymph was required for optimal outgrowth of Aplysia neurones. This communication assesses the species-specificity of conditioned medium (CM) for Helisoma, Lymnaea, Biomphalaria and Aplysia.

To make CM, isolated brains (two central ganglionic rings per ml for Helisoma, Biomphalaria or Lymnaea or one entire CNS per 10 ml for Aplysia) were placed in serum-free 50% Liebowitz medium (L-15) for 72 h with the salts appropriate for each species. For experiments with Helisoma, Biomphalaria or Lymnaea, salt concentrations of the media were: NaCl, 40 mM; KCl, 1.7 mM; CaCl₂, 4.1 mM; MgCl₂, 1.5 mM; 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, pH 7.3]. For Aplysia, salt concentrations were: NaCl, 460 mM; KCl, 10 mM; CaCl₂, 11 mM; MgCl₂, 55 mM; 5 mM-Hepes (pH 7.3). Isolated neurones for culture were obtained

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from Helisoma, Biomphalaria, Lymnaea and Aplysia basically as described previously (Wong et al. 1981). Neurones were dissociated by enzymatic digestion of dissected ganglia in 0.1% trypsin for 1.5 h. Enzymatic digestion was stopped by using 0.1% trypsin inhibitor for 15 min, and followed by desheathing of connective tissue and repeated trituration with plastic Pasteur pipettes. These mass-dissociated cells were transferred and cultured on polylysine-coated culture dishes in either defined medium or in CM.

To assess whether production was species-specific, CM produced by brains from each of the four species was placed in separate polylysine-coated culture dishes and plated with Helisoma neurones. For the dishes receiving Helisoma, Biomphalaria or Lymnaea CM, Helisoma neurones were plated directly since each medium contained the same salt concentrations. However, since the salt concentration for Aplysia CM is different from that of Helisoma, we took advantage of the fact that conditioning factor(s) binds to polylysine-coated surfaces (Wong et al. 1981). Thus Aplysia medium was discarded after covering the polylysine-coated surface for 24 h, and replaced with Helisoma defined medium before Helisoma neurones were plated.

Extensive neurite outgrowth of Helisoma neurones was obtained in the CM of each of the species tested (Fig. 1). As reported earlier (Wong et al. 1981), our primary criterion for outgrowth was a quantitative comparison of percentages of neurones sprouted (neurite length greater than the soma diameter) in defined medium vs experimental groups in CM. When the level of outgrowth was not significantly different on a quantitative basis, qualitative assessments determined whether outgrowth of the experimental groups was more extensive than in control groups (mostly spherical cells; Wong et al. 1981). Elaborate outgrowth, as in Fig. 1, was never seen in control dishes of defined medium.

Quantification of the growth-promoting activity (Fig. 2) showed that CM from Helisoma, Biomphalaria and Lymnaea brains significantly enhanced the outgrowth of Helisoma neurones (P < 0.05). However, surface adsorbed material (SAM) produced somewhat variable results. While Helisoma CM was highly significant (P < 0.001) in stimulating outgrowth, Helisoma SAM was significant to a lesser degree (P < 0.02). Similarly, Aplysia SAM, despite clearly fulfilling our more qualitative criterion for outgrowth, was only significant at a level of P < 0.1. One explanation for the low level of growth in Aplysia SAM is that the concentration of brains per unit medium for Aplysia was only a gross approximation, while that for Biomphalaria and Lymnaea was similar to that of Helisoma by virtue of similar animal sizes. An interesting possibility is that the phylogenetic distance between Aplysia and Helisoma may affect the ability of Aplysia CM to promote outgrowth in Helisoma neurones.

The above data show that outgrowth of Helisoma neurones can be promoted by a factor(s) from the brains of other molluscan species. This suggested the converse experiment. Because Helisoma CM has been extensively characterized (Wong et al. 1981; Barker, Wong & Kater, 1982), we used it to test whether a factor(s) was also necessary to promote the growth of neurones of other species. These experiments were complicated by the fact that the isolation procedures and growth conditions for neurones from different species are often different (cf. Dagan & Levitan, 1981; Mooney & Waziri, 1982). In two experiments in which we succeeded in growing...
Fig. 1. Phase contrast photomicrographs of mass dissociated *Helisoma* neurones that were cultured in medium conditioned by central ganglia of either *Lymnaea*, *Biomphalaria* or *Aplysia*, as well as *Helisoma*. Neurones were initially plated as spheres on polylysine-coated culture dishes, in 72h conditioned medium (CM) or surface adsorbed conditioned medium (SAM) and photographed 4 days after plating.
Neurite outgrowths from cultured molluscan cells

Fig. 2. Summary of species specificity of conditioned media. Cultures in conditioned medium (CM) or surface adsorbed conditioned medium (SAM) were scored as the relative percentage of adhering neurones which had neurites of a length greater than the soma diameter. Experiments were compared to controls (defined L-15 medium) after 4 days in culture. Differences from control were significant for Helisoma CM (P<0.001) and SAM (P<0.02), Biomphalaria CM (P<0.003) and Lymnaea CM (P<0.05). The result for Aplysia SAM approached significance (P<0.1). Error bars on each histogram represent S.E.M. (N = 3–8).

neurones of Biomphalaria in Helisoma CM there was significant (P<0.05) and elaborate neuronal growth compared to defined medium. This growth was indistinguishable from the growth of Helisoma neurones in Helisoma CM or growth of Biomphalaria neurones in Biomphalaria CM. All attempts to grow Lymnaea (four experiments) and Aplysia (two experiments) neurones in Helisoma CM failed to produce any significant numbers of cells with neurite outgrowth (P > 0.1). Nonetheless, Aplysia and Lymnaea neurones plated on their respective CM did produce neurite outgrowth which was several times more extensive than that seen in control conditions. The number of such neurones was small, however, and not suitable for quantitative analysis. However, by our qualitative criterion these two species required CM for outgrowth. These collective observations demonstrate that for Helisoma, Biomphalaria and perhaps Lymnaea and Aplysia, not only can their ganglia produce CM, but additionally, their neurones, when isolated in cell culture, require a CM for elaborating complex neuronal geometry.

The differences in growth of isolated molluscan neurones in vitro may be attributed
to several factors: (1) the dissociation procedures used; (2) the presence of factors such as sera, haemolymph or CM which are added to defined medium; or (3) the ganglionic origin and type of cell that is isolated (e.g. bag cells vs buccal ganglia). It is not surprising that Chen et al. (1971) failed to observe any neurite outgrowth in their cultures since the culture media consisted only of sea water. In agreement with our finding that CM stimulates neurite outgrowth is that in all other studies, with the exception of Kaczmarek et al. (1979), the addition of sera (Kostenko, Geletyuk & Veprintsev, 1974; Geletyuk, 1977; Dagan & Levitan, 1981; Proshansky et al. 1981), haemolymph (Kostenko et al. 1974; Proshansky et al. 1981), or brain extract (Kostenko et al. 1974) may have enhanced neurite outgrowth. An additional possibility is that Dagan & Levitan (1981) and Kaczmarek et al. (1979) may have released a factor(s) directly into culture media when ganglia were dissociated in the culture dishes. The observation by Kostenko et al. (1974) that the vitality and outgrowth of neurones is always better in an unwashed suspension is consistent with this view. The factor(s) may be similar to the ones that are added to defined medium by other investigators (Kostenko et al. 1974; Wong et al. 1981; Proshansky et al. 1981). Taken together these observations suggest that within the central ganglia of gastropods there is an endogenous supply of related neurite growth-promoting factor(s).

Our results clearly indicate that for closely related species (e.g. Helisoma and Biomphalaria) the growth-promoting activity of CM may be highly conserved and cross-species active. The growth-promoting activity of Lymnaea CM may be active across species in respect to its ability to support growth of Helisoma neurones. However, no outgrowth was observed in the complementary experiment of growing Lymnaea neurones on Helisoma CM. Likewise, the growth-promoting activity in CM produced by Aplysia, an even more distantly related marine gastropod, is also diminished, and is not cross-reactive when Aplysia neurones are plated on Helisoma SAM dishes. The reduced growth of both Lymnaea and Aplysia may be related to the phylogenetic distance between Helisoma and these two species. In addition to the question of species-specificity of CM, we have shown that Biomphalaria, Lymnaea and Aplysia neurones also appear to depend on their respective CM for growth.

The results of this study demonstrate that: (1) Helisoma, and three other species of molluscs require a conditioning factor(s) for neurite outgrowth; (2) brains from each of four species contain, or are capable of producing, such a factor(s); and (3) the mechanism of action of CM for each species may be through a common component or mode of action present in each CM. These data taken together suggest that gastropod molluscs may be capable of regulating the extensiveness of neuronal outgrowth by factors produced by their own nervous systems.

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Neurite outgrowths from cultured molluscan cells


