An attempt to examine the effect of prostaglandin E_2 and bradykinin on substance P containing neurons in grafts of dorsal root ganglia from chick embryos

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An Attempt to Examine the Effect of Prostaglandin E_2 and Bradykinin on Substance P Containing Neurons in Grafts of Dorsal Root Ganglia from Chick Embryos.

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Using grafts of dorsal root ganglia (DRG) of chick embryos, we tried to examine the effect of prostaglandin E_2 (PGE_2) and bradykinin (BK) on the substance P (SP) in primary sensory neurons by PAP immunohistochemistry.

The immunopositive neurons in the grafted DRG were composed of about 27 % after incubation in Krebs-Ringer solution, 32 % in BK, 21 % in PGE_2 and 20 % in BK+PGE_2, whereas those in the excised DRG were composed of 5 % in Krebs-Ringer, 7 % in BK, 7 % in PGE_2 and 9 % in BK+PGE_2. Although these data are rather insufficient to support the idea that BK and PGE_2 might subserve the release of SP, this preliminary experiment indicates that the grafts of DRG become useful tools for examining release regulation, biosynthesis and metabolism of SP in primary sensory neurons.

Key words: graft, chick embryo, dorsal root ganglion, substance P, prostaglandin E_2, bradykinin

Introduction

The SP present in a large proportion of avian DRG is suspected of being an excitatory transmitter or a modulator in primary sensory neurons. For nociception, in particular, stimulation of such sensory nerves seems to produce the release of SP from both central and peripheral terminals (Muramatsu, '87; Ueda et al., '85; Olgart et al., '77).

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Although the identity of sensory neurotransmitters is still uncertain, SP fibers projecting to the central side may act for excitation of pain, and SP fibers projecting to the peripheral side may be involved in the neurogenic inflammation process associated with injury and pain (Ueda et al., '85 ; Olgart et al., '77).

Concerning the ontogenesis of sensory neurons in chick DRG, several studies have been made recently (New & Mudge, '86 ; Fontaine-Perus et al., '85; Carr & Simpson, '78). The large sensory neurons in the lateroventral region arise earlier than the small ones in the mediodorsal region. The appearance of SP in small DRG neurons was first confirmed in chick embryos on embryonic day 5 (E5), before they came into contact with their peripheral targets. By E7, the SP positive small neurons had increased in number in the mediodorsal region, but the larger ones remained SP negative.

Undifferentiated cells are no longer detected after E12, and from this stage onward mediodorsal neurons became to show a high density of immunoreactive granules in the perinuclear spaces. It is said that such SP positive small neurons represent approximately 25 % of the total number of neurons in the DRG (New & Mudge, '86; Schwartz & Costa, '79 ; Hökfelt et al., '75).

Recently, PGE_2 in the homogenate of chick DRG was clearly analyzed by electrophoresis (Vesin & Droz, '88, unpublished data). Other biochemical studies have suggested that bradykinin activates the biosynthesis of PGs in various tissues which in turn enhance the release of SP from nerve terminals (Diener et al., '88 ; Ueda et al., '85 ; Juan et al., '84). Thus, we supposed that PGE_2 in DRG neurons might serve to regulate some transmitter function of SP. Since SP is a putative transmitter, it might be so sensitive as to be released immediately from the injured neurons. Therefore, in this experiment we tried to use the grafted DRG in order to examine the effect of BK and PGE_2 on the SP containing neurons.

It is now well established that the survival, maturation and growth of neurons in culture can be controlled by neurotrophic factors synthesized by their targets in limited amounts (Bossart et al., '88; Philippe et al., '88). Extension of neuritic processes from the dissociated DRG neurons has been confirmed within 55 hrs in cell culture (Barakat & Droz, '87) and SP immunoreactivity was detectable in vitro after 3 days in culture (Ernsberger & Rohrer, '88). Based on their data in cell culture, we considered that the grafted DRG neurons can recover from the injury enough to exhibit SP immunoreactivity within 4 days after transplantation.

Materials and Methods

Chick eggs were incubated at 38 ° C in a humid atmosphere in a draft incubator. Under a stereomicroscope, four DRG at the lumbosacral region and a piece of skin of the thigh were dissected from a donor on E12 in the physiological saline (Fig. 1(1)). Together they were transplanted onto the chorioallantoic membrane of a host under sterile conditions on E9 (Fig, 1(2)). The eggs were sealed by a cover glass and wax and then incubated again for 4 days. A whole graft of DRG and skin was carefully removed from the chorioallantoic membrane and immediately transfered into one of the following mediums (Fig. 1 (3)). a) basic medium: Krebs-Ringer solution containing 0.012 M glucose, aired with gas of O_2/ CO_2 b)10^{-6} M BK in the basic medium c) 0.33×10^{-6} M PGE_2 in the basic medium d)10^{-6} M BK + 0.33×10^{-6} M PGE_2 in the basic medium. They were then incubated for 10 min at 37 ° C and the grafts were fixed in modified PLP solution overnight at 4°C (Mclean & Nakane, '74). Thereafter, the grafts were embedded in paraffine. Serial sections of 6 μm in thickness were pretreated with 5 % H_2O_2 for 10 min in order to

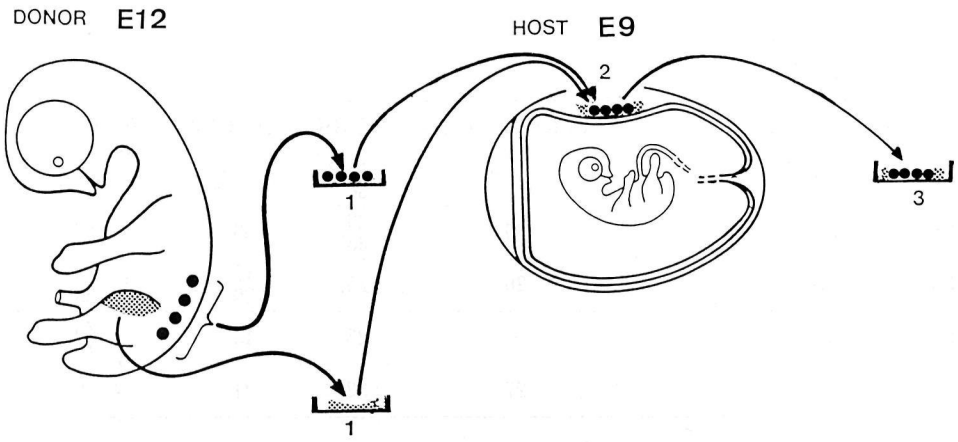

Fig.1

Illustration of experimental procedures. See text.

block endogenous peroxidase activity. SP immunohistochemistry was performed by PAP method.

First incubation : mouse monoclonal antibodies against SP diluted 1/500 in 0.1 M PBS (osmolarity : 317mOsm/kg) containing 0.5% Triton X-100, for 48 hs in a humid box at 4°C. Second incubation : rabbit anti-mouse I gG diluted 1/40 in 0.1M PBS, for 1h at room temperature Final incubation : PAP complex diluted 1/100 in 0.1 M PBS, for 1 h at room temperature. The sections were visualized by 0.06% of 3.3'-diaminobenzidine tetrahydrochloride solution with 0.01 % H_2O_2. The sections were slightly counter stained with toluidine blue and the neurons showing a nucleus were counted in every third section.

Results and Discussion

It was obvious that the cell size of the grafted DRG neurons was 20 % smaller than that of the excised DRG ones on E16. Within the grafts, the initial localization of small and large neurons was not clear because of the random cell migration. Large cells from the lateroventral region and small ones from the mediodorsal region were intermingling in every graft. In fact, some neurons seemed to degenerate but most of them recovered from the neuronal injury, extending their processes to the grafted skin (Fig, 2 A, B). Probably the presence of targets in the graft make it easy for them to maintain survival and promote induction of SP immunoreactivity in the grafted DRG (Bossart et al., '88; Philippe et al., '88).

In each graft, more than 500 neurons were counted and the percentages of SP immunopositive cells present within the total number of neurons are summarized in Table I. About 27 % of the neurons were immunopositive in the case of incubation in Krebs-Ringer. This is quite close to the results reported by other authors in normal development (25 % in rat DRG).

Unfortunately, however, the percentages of SP immunopositive neurons in each graft were quite variable after incubation even in the same conditioned medium. This might reflect the variation in the neuronal regeneration. The grafts of DRG incubated in BK showed a high percentage (about 32 %). In the case of those incubated in BK, we had the impression that the immunoreaction was much stronger than those in the others (Fig, 2). When the grafts were incubated in the mediums containing PGE_2 and BK+PGE_2, percentages

Table. I

Percentages of SP immunopositive cells present within the total number of neurons counted in the grafted DRG.

conditioned medium	a) Krebs-Ringer	b) BK	c) PGE2	4) BK+PGE2
Exp 1 (3 grafts)	25 %	40 % 30	−%	−%
Exp 2 (6 grafts)	30	39 35	33	26 20
Exp 3 (6 grafts)	26	25	15 20	10 28
Exp 4 (4 grafts)	−	25	15	25 10
average	27	32	21	20

Table. II

Percentages of SP immuopositive cells present within the total number of neurons counted in the excised DRG at E 16.

conditioned medium	a) Krebs-Ringer	b) BK	c) PGE2	d) BK+PGE2
Exp 1 (3 DRG)	3 %	−%	6 %	9 %
Exp 2 (4 DRG)	7	7	8	8
average	5	7	7	9

slightly decreased to 21 % and to 20 % respectively. It might indicate that PGE_2 stimulated the release of SP from the grafted DRG neurons.

As shown in Table II, only 5 % of the excised DRG neurons were immunopositive after incubation in the Krebs-Ringer solution, and 7 % in BK, 7 % in PGE_2 and 9 % in BK+PGE_2. These percentages were distinctly low in comparison with those in grafted DRG even after incubation in Krebs-Ringer solution. Furthermore, immunoreaction of SP positive neurons in the excised DRG was much weaker than that in the grafted DRG. Therefore it is very difficult to conclude the effect of PGE_2 and BK on SP neurons. Probably, when DRG were dissected from an embryo, the injured neurons immediately lost the SP content and reduced their immunoreactivity seriously. However, we noted that a few small neurons located in the periphery of excised DRG displayed a prominent SP immunoreacton around the Golgi apparatus (Fig, 3 A, B). Such a strong immunoreaction around the Golgi apparatus was recognized only in the excised DRG neurons incubated in BK and BK + PGE_2 solutions. This might arise as the result of incubation in BK containing medium.

Although it is said that BK stimulates the synthesis of PGE_2 which subsequently stimulates the release of SP, sufficient evidence was not found in this experiment. Without bioassay data, it is difficult to determine the effect of BK and PGE_2 on SP neurons. Nevertheless, in this preliminary experiment, we emphasize that the grafted DRG is an useful tool for studying release regulation, biosynthesis and metabolism of SP in the primary

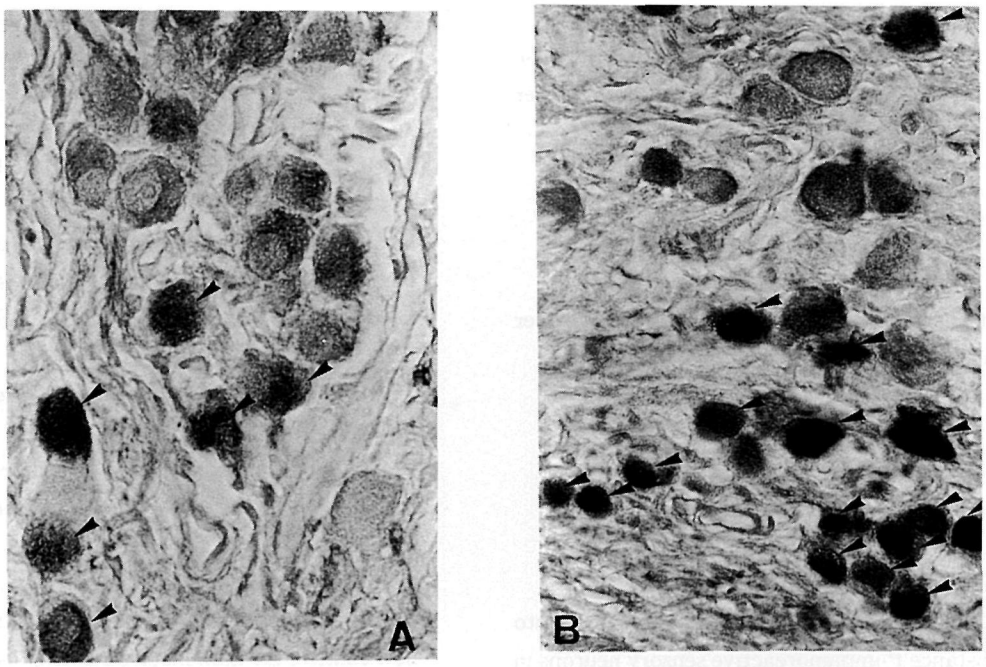

Fig. 2 A : SP immunoreaction of a grafted DRG incubated in Krebs-Ringer solution. SP immunopositive neurons are marked by arrow heads. Newly formed fibers are also immunopositive. ×650 **B :** SP immunoreaction of a grafted DRG, incubated in the BK added solution. Small neurons are SP immunopositive (arrow heads) but larger ones are negative. ×400

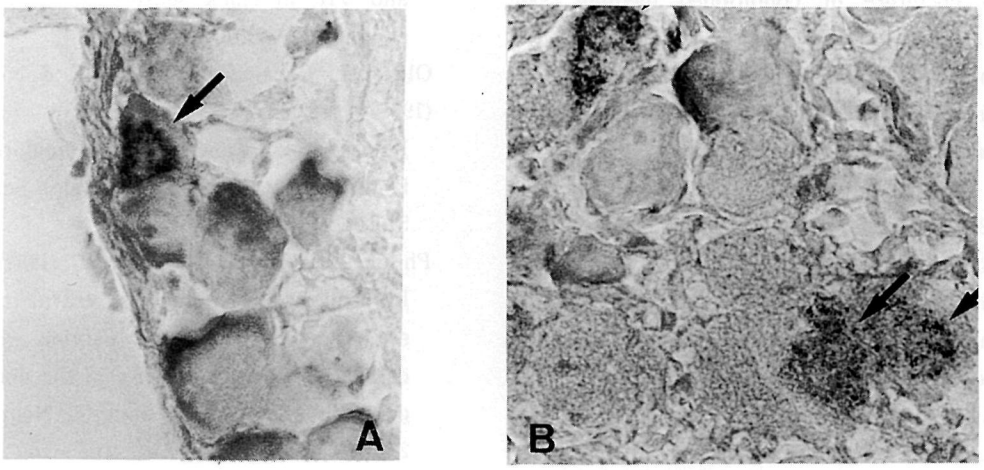

Fig. 3 A : SP immunoreation of an excised DRG on E16, incubated in the BK + PGE_2 added solution. A small neuron marked by an arrow exhibits intensifined SP immunoreaction around the Golgi area. ×650 **B :** SP immunoreaction of an excised DRG on E16, incubated in the BK + PGE_2 added solution. Arrows indicate SP positive neurons showing granular or reticular reaction products around the Golgi area. ×650

sensory neurons. In order to determine a precise effect of PGE_2 and BK on SP neurons, further experiments are required.

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鶏胚脊髄神経節の移植片を用いてSP含有神経細胞に対するPGE$_2$とBKの影響を観察する試み

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鶏胚脊髄神経節の移植片を用いて，サブスタンスP(SP)含有知覚神経細胞に対するプロスタグランジンE_2 (PGE_2)及びブラジキニン(BK)の影響を，免疫組織細胞化学的に観察した。

12日胚から腰仙髄部の脊髄神経節を摘出し，大腿部の皮膚と共に9日胚の漿尿膜に無菌状態で移植した。4日間の孵卵後，移植片を傷つけないように摘出し，Krebs-Ringerの基本液，10^{-6} M BK添加の液，0.33×10^{-6} M PGE_2添加の液，10^{-6} M BKと0.33×10^{-6} M PGE_2添加液の4液の中でそれぞれ10分間培養し，PLP法で固定してパラフィンに包理した。連続切片は，脱パラ後，5%のH_2O_2で前処理し，500倍希釈の抗SPモノクローナル抗体を用いて4°Cで2日間反応させPAP法で可視化した。移植片中の総神経細胞に対するSP陽性細胞の割合は，平均すると，Krebs-Ringer液で培養したものでは27%，BK液では32%と少し高く，PGE_2液では21%，BK + PGE_2液では20%と低下の傾向を示した。一方，16日胚の脊髄神経節を摘出して直ちにこれらの液で培養した場合には，Krebs-Ringer液で5%，BK液で7%，PGE_2液で7%，BK + PGE_2液で9%といずれも低く，反応も移植片の場合に比べて著しく弱かった。細胞の損傷によるSP放出の為と思われる。

この予備実験は，一次知覚神経に含まれるSPの放出調節，産生，代謝などに与える薬物の影響を研究する上に，移植片を用いることの有効性を示す。