

Differential effects of the protein kinase inhibitor K-252a on the in vitro survival of chick embryonic neurons

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The effects of the protein kinase inhibitor K-252a on the in vitro survival of different populations of chick embryonic neurons were tested. Following dissociation from the corresponding ganglia by trituration, the neurons were cultured on laminin-coated dishes in both the presence and absence of their respective neurotrophic factor. K-252a alone promoted long-term neuronal survival of dorsal root ganglion (DRG) and ciliary ganglion (CG) neurons in a dose-dependent fashion. No such effect was seen with sympathetic ganglion (SG) neurons. In addition, K-252a did not interfere with nerve growth factor (NGF)- or ciliary neurotrophic factor (CNTF)-mediated survival of DRG or CG neurons, respectively, but completely blocked the NGF- and CNTF-induced survival of SG neurons. High potassium-induced survival of SG neurons was not affected by K-252a. These results point to differences between various neuronal populations in the signal transduction mechanism for neurotrophic factors.

It is now widely recognized that the survival of vertebrate embryonic neurons depends on target-derived neurotrophic factors, nerve growth factor (NGF) being so far the most studied molecule [3,19]. Despite extensive studies, still little is known about the signal transduction of neurotrophic factors, including NGF, resulting in neuronal survival and/or fiber outgrowth. Recent results obtained in PC12 cells [4,10,18] and cultured neurons [5] support the concept of a central role for the *ras* proto-oncogene protein in the signalling cascade initiated by the binding of neurotrophic factors to their specific receptors. However, few data are available regarding the signalling steps upstream and downstream of the *ras* protein.

One of the major difficulties for such studies has been the lack of agents specifically blocking the actions of neurotrophic factors. Recently, a new protein kinase inhibitor of microbial origin, K-252a [14], has been found to inhibit specifically a number of NGF-induced responses in the NGF-sensitive pheochromocytoma cell line PC12

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[11,15]. In addition, K-252a has been reported to inhibit both NGF-induced neurite outgrowth in cultured chick embryo dorsal root ganglion (DRG) cells [17], and NGF-dependent increase in neurofilament protein expression in cultured human embryonic DRG neurons [7]. In order to further examine the effects of K-252a, I tested this substance on 3 different neuronal populations isolated from the chick embryo.

Chick dorsal root ganglion (DRG) neurons at embryonic day 9 (E9), ciliary ganglion (CG) neurons at E8 and sympathetic ganglion (SG) neurons at E12 were isolated from the corresponding ganglia and cultured using previously described methods [5,8]. After preparation, the ganglia were trypsinized (0.2% trypsin in PBS for 30 min) and dissociated by mild trituration. The cell suspension was preplated as described [16] on Nunc tissue culture dishes. The neuron-enriched cell suspensions were plated on Costar 24-well plates (1.6 cm well diameter) which had been coated sequentially with poly-DL-ornithine (0.5 mg/ml, Sigma) and laminin (5 μ g/ml, BRL) as described [6,9]. The cell density was 10,000 cells/well (8,000 for ciliary neurons). Non-neuronal cells comprised < 5% of the total population. Immediately after plating, factors were added to the appropriate wells at the following final concentrations: NGF 20 ng/ml, CNTF 10 ng/ml, K⁺ 35 mM, K-252a 50–800 nM. NGF and CNTF were purified as described [12,13]. The cultures were maintained with F14 medium (Gibco) containing 10% heat-inactivated horse serum (Boehringer Mannheim) at 37°C and 3.5% CO₂ in a humidified environment. 24 to 48 h after plating, approx. 1/8 of the well surface was scanned for the presence of phase-bright cells. This procedure was repeated after 8 and 15 days to establish long-term survival rates. Cell counting was performed on a Leitz inverted microscope, magnification 125 \times .

On E9 DRG neurons, a dose-dependent survival effect of K-252a *alone* was observed (Fig. 1A). The highest survival rate was found at a concentration of 800 nM and was virtually identical with the survival levels routinely obtained with NGF in this system. The NGF-induced survival was marginally impaired at K-252a concentrations \geq 200 nM. Morphologically, however, the DRG neurons cultured in the presence of K-252a showed less pronounced cell body hypertrophy as compared to the NGF-treated ones (Fig. 2A–C). In addition, neurite outgrowth induced by K-252a resulted in finer and more branched processes (Fig. 2B). The survival effect induced by K-252a persisted without significant decrease for over two weeks (data not shown).

Similar results were obtained with E8 ciliary ganglion neurons (Fig. 1B). Again, K-252a promoted neuronal survival in a dose-dependent fashion, and did not interfere with CNTF-induced survival. Morphological changes were comparable to those observed in DRG neurons (Fig. 2D–F). Again, neurite outgrowth and arborization were enhanced and the processes appeared thinner than in the control cultures.

Surprisingly, a completely different picture was observed using E12 sympathetic neurons. Here, K-252a had no intrinsic survival-promoting activity, but inhibited virtually completely the survival effects of NGF. The same result was observed with CNTF, which is also known to promote the *in vitro* survival of sympathetic neurons [2]. That the inhibitory action of K-252a was not due to a general toxicity of this

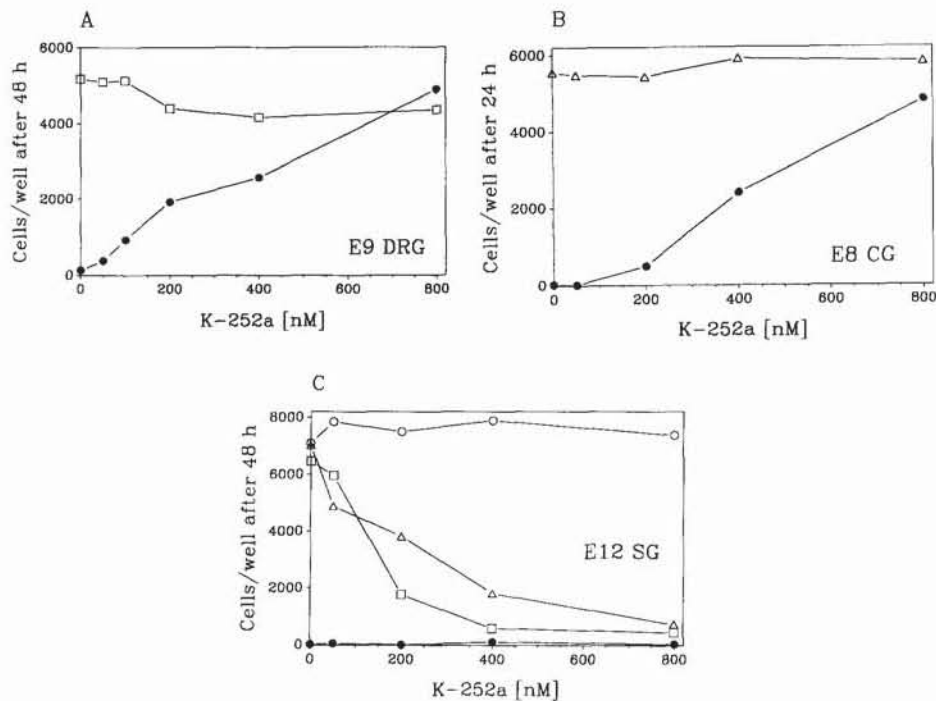


Fig. 1. Effects of K-252a on cultured chick embryonic neurons. Cells were cultured as described in the text and grown in the presence of the indicated K-252a concentrations only (filled circles) or together with 20 ng/ml NGF (empty squares), 10 ng/ml CNTF (empty triangles), or 35 mM K⁺ (empty circles) for 48 h (24 h for ciliary neurons). A: E9 DRG neurons. B: E8 ciliary neurons. C: E12 sympathetic neurons.

substance was demonstrated by the fact that survival induced by high (35 mM) K⁺ concentration was not affected (Figs. 1C and 3).

The data obtained with K-252a on sympathetic neurons are reminiscent of the effects of the methyltransferase inhibitor 5'-deoxy-5'-methylthioadenosine (MTA) on the same cells. Like K-252a, MTA at 3 mM was shown to specifically inhibit NGF-, but not high K⁺-induced survival of E12 sympathetic neurons [1].

The observation of the intrinsic K-252a survival activity on DRG and ciliary neurons was somewhat unexpected for two reasons: indeed, previous reports had described inhibitory effects of the action of NGF on DRG neurons by K-252a [7,17]. In addition, K-252a at concentrations > 200 nM is known to be toxic, at least on PC12 cells [15]. However, in previous reports dealing with the effects of K-252a on cultured neurons, no quantitative survival assessment was performed, and no data were shown regarding the effects of K-252a alone at concentrations > 50 nM.

At present, it is difficult to offer a satisfactory explanation for the discrepancy between the observations reported here and some previous reports [7,17]. The remarkable discrepancy between the effects of high concentrations of K-252a on either ciliary and sensory neurons on the one hand (promotion of cell survival) and on sympathetic neurons on the other (blockade of NGF- or CNTF-induced survival)

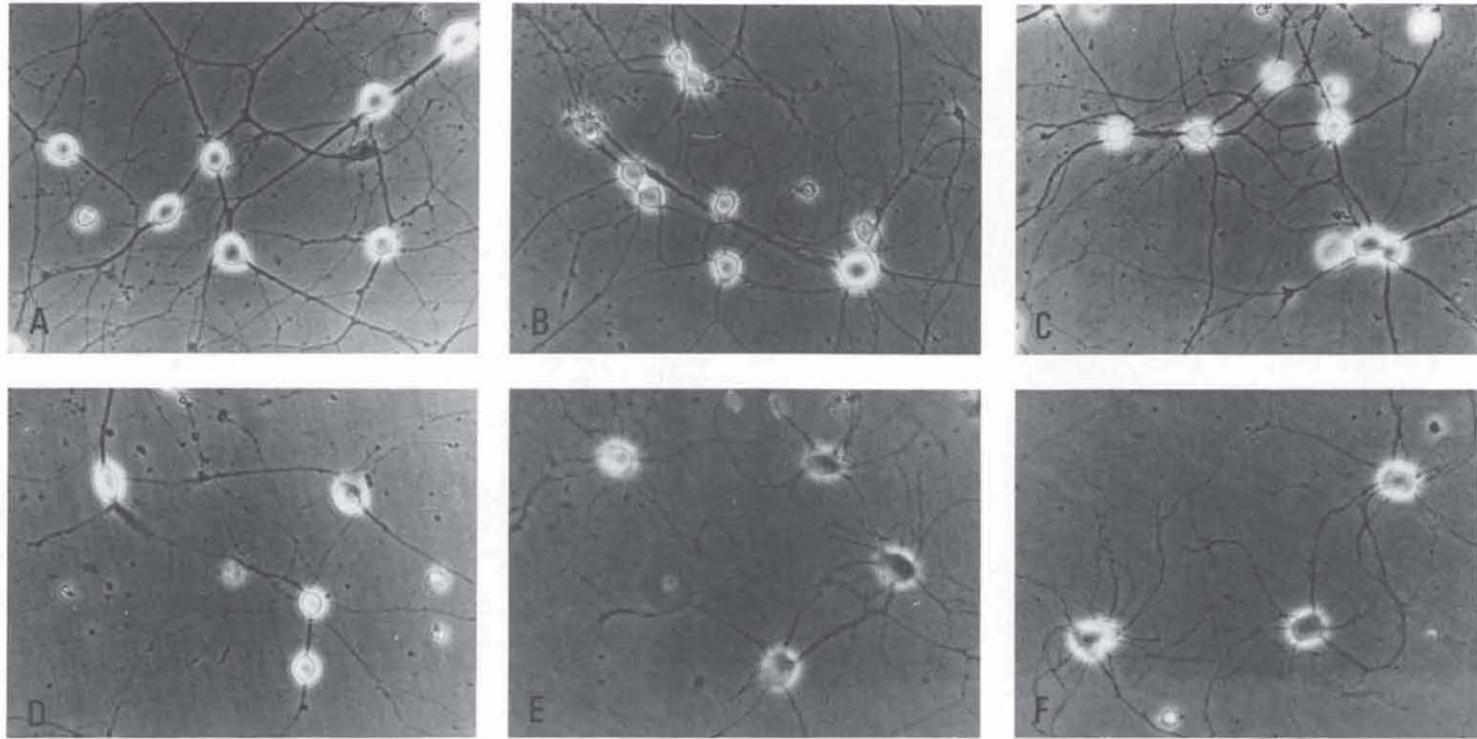


Fig. 2. Phase micrographs of E9 DRG (A–C) and E8 ciliary (D–F) neurons cultured for 48 h (24 h for ciliary neurons) in the presence of: (A) NGF (20 ng/ml), (B,E) K-252a (800 nM), (C) NGF + K-252a, (D) CNTF 10 ng/ml, (F) CNTF + K-252a. Note the smaller cell body size in B and C as compared to A and the pronounced neurite outgrowth in B, E and F. Magnification 290 × .

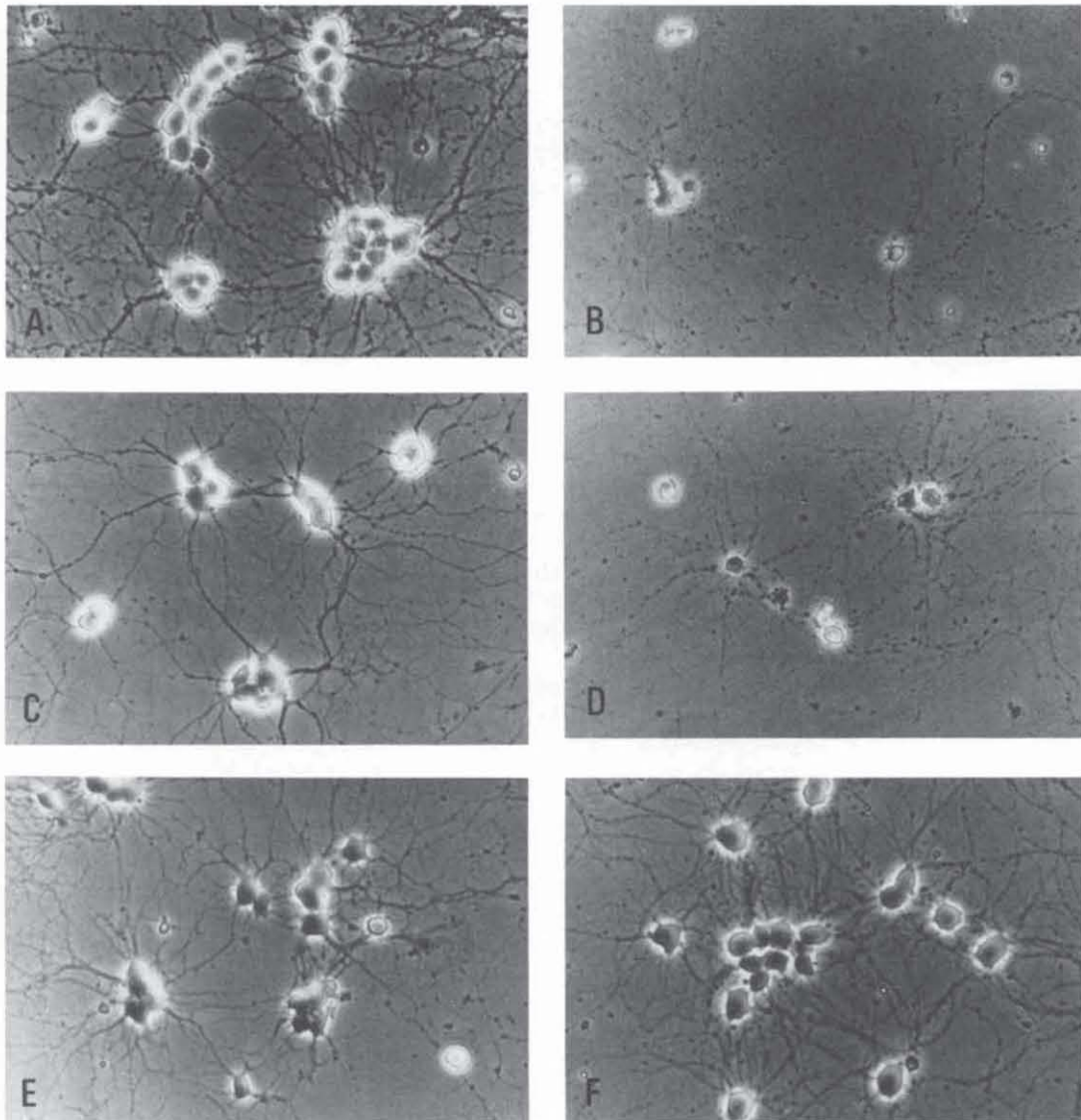


Fig. 3. Phase micrographs of E12 sympathetic neurons cultured for 48 h in the absence (A,C,E) or presence (B,D,F) of 800 nM K-252a. Factors added to the cultures were: NGF 20 ng/ml (A,B), CNTF 10 ng/ml (C,D) and K^+ 35 mM (E,F). Magnification 290 \times .

is unexpected and might point to differences in the intracellular mechanisms leading to neuronal survival in different neuronal populations. Thus, identifying the precise biochemical site and mode of action of K-252a could prove very useful for the elucidation of the signal transduction of neurotrophic factors.

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