The Neurotransmitter $\gamma$-Aminobutyric Acid Is an Inhibitory Regulator for the Migration of SW 480 Colon Carcinoma Cells

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Abstract
$\gamma$-Aminobutyric acid (GABA) is the inhibitory neurotransmitter in the brain, also playing a role in diseases like epilepsy. We now show that this inhibitory neurotransmitter can also reduce migratory activity in SW 480 colon carcinoma cells. GABA reduced the norepinephrine-induced migratory activity of these cells within a three-dimensional collagen matrix to spontaneous migration levels, as was analyzed by time-lapse videomicroscopy. This inhibitory effect of GABA was mediated by the serpentine receptor GABAB and was intracellularly transduced by a decrease of the cyclic AMP concentration. Cancer cell migration is thus regulated by neurobiological signals, opening new possibilities for pharmacological agonists in cancer therapy.

Introduction
Evidence is growing that the migration of tumor cells is not solely a consequence of genetic alterations but is regulated by a multitude of epigenetic factors. Chemokines, neurotransmitters, and other structurally unrelated ligands of serpentine receptors are known as important initiators of migratory activity (1). We have previously reported on the initiation of colon carcinoma cell migration by the neurotransmitter norepinephrine (2). In a further development of our studies on the influence of neurotransmitters on the migration of cancer cell migration, we investigated the effect of the inhibitory neurotransmitter GABA on the norepinephrine-induced migration of SW 480 colon carcinoma cells. GABA is the major inhibitory neurotransmitter of the central nervous system, where it has been shown to play a role in pathological conditions like epilepsy (3). GABA can, however, also be found in neural and nonneural tissues outside the brain (4, 5), including the gastrointestinal tract (6). Principally, GABA acts on two classes of cellular receptors: (a) the ionotropic GABAA and GABAC receptors are oligomeric chloride channels (6, 7); and (b) the metabotropic GABAB receptor is a member of the serpentine or seven-helices receptor family and is therefore related to chemokine receptors and catecholaminergic receptors, which both have been shown to be involved in the regulation of leukocyte and tumor cell migration (1, 8). Herein, we describe the signal transduction of the antitumor effects of norepinephrine and GABA on the regulation of tumor cell migration.

Materials and Methods
Cell Culture. The colon carcinoma cell line SW 480 (American Type Culture Collection, Manassas, VA) was maintained at standard conditions as described previously (2).
Cell Migration Assay. The cell migration assay was performed as described in detail previously (9). In short, cell locomotion within three-dimensional collagen lattices was recorded by time-lapse videomicroscopy overnight. For the analysis of migratory activity, 30 cells of each sample were randomly selected, and two-dimensional projections of paths were calculated by computer-assisted cell tracking in 20-min step intervals.

Measurement of Cellular cAMP. For the measurement of changes in the cellular cAMP, $6 \times 10^5$ cells were incubated for 20 min at 37°C with either medium alone or with 10 $\mu M$ norepinephrine, 100 $\mu M$ GABA, or the combination of both neurotransmitters. For positive control, the cells were incubated with 500 ng/ml cholera toxin or 500 ng/ml pertussis toxin (both Sigma, Deisenhofen, Germany) under the same conditions. After incubation, cells were lysed, and the cAMP level was measured using a cAMP enzyme-linked immunosassay system (Amersham Pharma Biotech, Buckinghamshire, United Kingdom) as described by the manufacturer.

Flow-Cytometrical Measurement of Cytosolic Calcium. For the investigation of changes in cytosolic calcium by treatment with norepinephrine or GABA, the SW 480 colon carcinoma cells were loaded with fluo-3.a.m. as previously described with minor modifications (10), and the calcium-induced fluo-3.a.m. fluorescence was measured immediately after addition of norepinephrine or GABA alone or in combination.

Results and Discussion
The Inhibition of Norepinephrine-induced Migration of Colon Carcinoma Cells by GABA Requires the Engagement of GABAB Receptors. Norepinephrine induces migration of SW 480 colon carcinoma cells (2). We now report that this induced tumor cell migration is completely inhibited by GABA (Fig. 1A). Norepinephrine induces migration from 42 ± 13% spontaneously locomoting cells to 63 ± 3%, whereas GABA alone had no effect (46 ± 4% locomoting cells) but abolished norepinephrine-induced migration (37 ± 3%). We investigated the receptor for the regulatory function of GABA by specific agonists (Fig. 1, B and C). We used baclofen as a specific agonist for GABAB receptors and isoguvacine for GABA receptors. Baclofen but not isoguvacine inhibited the norepinephrine-induced migration (Fig. 1, B and C). After treatment with baclofen, the norepinephrine-induced migration was reduced from 52 ± 5% down to 33 ± 4% locomoting cells, whereas treatment with isoguvacine had no effect (44 ± 3% locomoting cells with norepinephrine versus 45 ± 2% locomoting cells with norepinephrine and isoguvacine). Thus, the inhibitory function of GABA on the migration of these tumor cells is mediated by GABAB receptors.

The Inhibitory Effect of GABA Is Mediated by the Regulation of Cellular cAMP. It is well known from heart muscle cells that binding of norepinephrine leads to activation of stimulating G proteins (11). These G proteins regulate the activity of the adenyl cyclase, which generates the second messenger cAMP (12). In turn, cAMP binds to multiple effector molecules, which are involved in the regulation of the cytosolic calcium concentration. A key effector mole-
cule is the protein kinase A, which phosphorylates phospholamban. Phosphorylation of phospholamban leads to its release from sarcoplasmic/endoplasmatic reticulum calcium ATPase, which sequesters cytosolic calcium into intracellular stores (13).

In addition to the G protein-mediated signaling, Luttrell et al. (14) described an activation of PTKs via /H9252-arrrestin after /H9252-adrenoceptor engagement. We have shown that a PTK-dependent activation of the PLC is a crucial regulator for the norepinephrine-induced migration of colon carcinoma cells (2). Two second messengers are generated by the PLC: (a) inositol-1,4,5-trisphosphate, which opens intracellular calcium channels; and (b) diacylglycerol, which activates the PKC. In this context, it is important to stress that an activation of the PKC with the diacylglycerol analogue phorbol-12-myristate-13-acetate is a sufficient start signal for the induction of very high locomotor activity in colon carcinoma cells (15).

In our experiments on the regulatory signal transduction of the norepinephrine- and GABA-mediated effects on cell migration, we focused on the G protein-mediated regulation of cellular cAMP. Norepinephrine induced an increase of cellular cAMP by 45.2%, whereas GABA reduced the cellular cAMP concentration by 48.5% (Fig. 2A). The addition of GABA to norepinephrine-treated cells significantly reduced the norepinephrine-induced increase of cAMP. Therefore, an increase of locomotor activity is coupled to an increase of cellular cAMP, whereas a reduction of cellular cAMP decreases migratory activity.

Cells treated with dibutyryl-cAMP in combination with GABA and...
GABA inhibits SW 480 tumor cell migration

norepinephrine developed a migratory activity of 55 ± 18% locomoting cells, which was similar to norepinephrine alone (55 ± 15% locomoting cells), whereas cells treated with norepinephrine and GABA revealed a migratory activity of 36 ± 12% locomoting cells (Fig. 2B). Addition of dibutyryl-cAMP alone did not lead to an increase of migratory activity (27 ± 4% locomoting cells). This shows that a decrease of cAMP is a sufficient stop signal for the norepinephrine-induced migration, but an increase of cAMP is not a sufficient start signal. As pointed out above, we have shown previously that an activation of the PKCα alone is a sufficient signaling event for the norepinephrine-induced migration of SW 480 colon carcinoma cells, whereas no change of cAMP is observed with norepinephrine alone (Fig. 3). GABA reduces the cAMP concentration (Fig. 2A) and causes thereby a reduced calcium sequestration. This interruption of the previously described calcium cycling (10) results in the reduction of locomotor activity (arrow C in Fig. 4).

In conclusion, the induced migration of SW 480 colon carcinoma cells is inhibited by the neurotransmitter GABA. Our results lend themselves to the investigation of the possible use of GABAA receptors for the chemoprevention of metastasis development. Accordingly, Kawabata et al. (16) provided evidence for a preventive role of GABA in the frequency of azoxymethane-induced colonic adenocarcinoma in rats. Using this novel knowledge of neurobiological influences and the concerned intracellular pathways of tumor cell migration, it might be possible to develop strategies to delay or prevent metastasis formation either by blocking the involved receptors or by interfering with the intracellular signal transduction.

References


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Fig. 4. Model of the signal transduction pathways regulating the norepinephrine-induced locomotion and the inhibition of migration by GABA. This model is an extension of the pathway published in Ref. 2 (22). The norepinephrine-induced pathways, which are marked by the arrows A and B, lead to a release of calcium from the endoplasmatic reticulum and its sequestration, thereby promoting a calcium cycling within the cell. The GABA-induced pathway, which is marked by the arrow C, interrupts this cycling by inhibiting the calcium sequestration. AC, adenylyl cyclase; PLB, phospholamban.
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