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GABA induces terminal differentiation of *Dictyostelium* through a $GABA_B$ receptor

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When prespore cells approach the top of the stalk in a *Dictyostelium* fruiting body, they rapidly encapsulate in response to the signalling peptide SDF-2. Glutamate decarboxylase, the product of the *gadA* gene, generates GABA from glutamate. *gadA* is expressed exclusively in prespore cells late in development. We have found that GABA induces the release of the precursor of SDF-2, AcbA, from prespore cells. GABA also induces exposure of the protease domain of TagC on the surface of prestalk cells where it can convert AcbA to SDF-2. The receptor for GABA in *Dictyostelium*, GrlE, is a seven-transmembrane G-protein-coupled receptor that is most similar to $GABA_B$ receptors. The signal transduction pathway from GABA/GrlE appears to be mediated by PI3 kinase and the PKB-related protein kinase PkbR1. Glutamate acts as a competitive inhibitor of GABA functions in *Dictyostelium* and is also able to inhibit induction of sporulation by SDF-2. The signal transduction pathway from SDF-2 is independent of the GABA/glutamate signal transduction pathway, but the two appear to converge to control release of AcbA and exposure of TagC protease. These results indicate that GABA is not only a neurotransmitter but also an ancient intercellular signal.

KEY WORDS: gadA, GrlE, SDF-2, Sporulation, GPCR

INTRODUCTION

The efficiency and timing of sporulation in *Dictyostelium discoideum* is regulated by a secreted peptide, SDF-2, that binds to the receptor histidine kinase DhkA (Wang et al., 1999). The sequence of this 34 amino acid peptide is very similar to that of the mammalian neuropeptide DBI, which binds to the $GABA_A$ ionotropic receptor at the benzodiazapine-binding sites that modulate the affinity to GABA. SDF-2 is processed from the precursor protein AcbA by cleavage at sites recognized by trypsin. This 88 amino acid precursor binds acylCoA as does the precursor of DBI (Anjard and Loomis, 2005). In a bioassay with monolayers of cells overexpressing the catalytic subunit of the cAMP-dependent protein kinase PKA, developing cells respond to SDF-2, as well as to DBI and diazepam, by rapidly encapsulating. The response to each is mediated by the histidine kinase receptor DhkA.

Proteolytic processing of AcbA to generate the signal peptide SDF-2 is dependent on the prestalk specific ABC-protease TagC and occurs in the intercellular space (Anjard and Loomis, 2005). AcbA is found exclusively in prespore cells during late development and must be released before it is processed on the surface of prestalk cells. Prespore cells are stimulated to rapidly release AcbA when primed with SDF-2. Likewise, SDF-2 triggers exposure of the protease activity of TagC on prestalk cells such that AcbA can be processed. Unprimed cells do not generate SDF-2 when presented with recombinant AcbA. Together, these responses amplify and relay the signal. However, it is not clear what initiates the original release of SDF-2 and the exposure of the TagC protease domain.

Dictyostelium has been shown to produce GABA in both growing and developing cells (Ehrenman et al., 2004). Two genes encoding the enzyme that converts glutamate to GABA, glutamate decarboxylase, can be recognized in the *Dictyostelium* genome. Microarray-based experiments have shown that one of these genes, *gadA*, is expressed only after 10 hours of development and its mRNA accumulates to a peak at 18 hours when the cells are initiating fruiting body formation (Iranfar et al., 2001). The other gene, *gadB*, is expressed in growing cells and its mRNA continuously decreases during development (Van Driessch et al., 2002; Iranfar et al., 2003). GadB is likely to be responsible for the metabolism of glutamate via the GABA shunt to succinate during growth. As *gadA* is exclusively expressed in prespore cells starting at 10 hours of development, as determined by microarray analyses and in situ hybridization (Iranfar et al., 2001; Maruo et al., 2004), GadA may play a late developmental role. We disrupted *gadA* by homologous recombination and found that the mutant cells grow and develop well, forming normally proportioned fruiting bodies, but the number of viable spores was reduced to about half that found in wild-type strains. As mutants lacking AcbA or DhkA are also impaired in sporulation, the similar phenotype of $gadA^-$ cells prompted us to investigate the role of GABA in generation of SDF-2. We found that addition of 1 nM GABA to developed cells effectively triggered the rapid release and processing of AcbA to generate the signal peptide SDF-2.

Signaling in the central nervous system is mediated to a large extent by glutamate and GABA. These intercellular signals and their receptors are found in *C. elegans*, *Drosophila* and all vertebrates but have not been found in yeast or protists. Glutamate and GABA activate ionotrophic and metabotropic receptors on the surface of neurons to initiate and modulate neurotransmission and also play roles in peripheral tissues. Careful inspection of the coding capacity of the *Dictyostelium discoideum* genome found that ionotropic receptors are absent but that there are 15 genes encoding homologs of glutamate and GABA metabotropic receptors (Eichinger et al., 2005; Hereld, 2005). Although all of these proteins are predicted to be seven transmembrane G-protein-coupled receptors (GPCRs), only one, GrlE, shows significant similarity in the ligand binding domain to the $GABA_B$ family. The others show similarity in the transmembrane and cytoplasmic G protein-binding regions but not in the ligand-binding domain. We generated a $grlE^-$ null strain by

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homologous recombination and found that cells of this strain do not produce SDF-2 in response to GABA, suggesting that GrlE is the GABA receptor in *Dictyostelium*.

MATERIALS AND METHODS

Chemicals

GABA (γ-aminobutyric acid), L-glutamate and cAMP were supplied by Sigma. SDF-2 was synthesized as previously reported (Anjard and Loomis, 2005). The $GABA_B$ receptor antagonists CGP 55845 and CGP54626 were purchased from Tocris, Ellisville, MO. The PI3 kinase inhibitor LY294002, AKT inhibitor IV and VIII, the PKA inhibitor H89 and the phospholipase inhibitor U73122 were purchased from Calbiochem.

Cells and bioassay

The wild type strain AX4, the *pkaC* overexpressing strain KP and its derivative *dhkA*$^-$/K, the PI3 kinase double mutant *pik1*$^-$ *pik2*$^-$, and the *pkbR1*-null mutant have been previously described (Knecht et al., 1986; Anjard et al., 1992; Anjard et al., 1998; Buczynski et al., 1997; Meili et al., 2000). The *acaA*$^-$K strain, which lacks the G-protein-coupled adenylyl cyclase and overexpresses *pkaC* has been previously characterized (Anjard et al., 2001). The Gα9-null strain (*gpaI*$^-$) has been previously described (Brzostowski et al., 2004). Cells were grown in HL5 at 22°C and developed on buffer-saturated filters or on non-nutrient agar (Sussman, 1987; Anjard et al., 1998). It was essential to develop cells of the *pik1*$^-$ *pik2*$^-$ and *pkbR1*$^-$ mutant strains on non-nutrient agar to get the majority to pass the tight aggregate stage and proceed through morphogenesis. Fruiting bodies were washed, and SDF-1 and SDF-2 separated using ion-exchange resins before being assayed as previously described (Anjard et al., 1998).

To generate *gadA*$^-$ null strains by homologous recombination, a 1419 bp genomic fragment starting 276 bp after the initiation codon of GadA was amplified by PCR and cloned in the pGEMT-EASY vector (Promega A1360). The BSR cassette from pBSR519 (Puta and Zeng, 1998) was cloned into the *Hin*dIII sites of pGEMT-EASY-GadA, resulting in the loss of 357 bp of *gadA*-coding sequence. For gene disruption, 10 μg of the plasmid was linearized with *Not*I before electroporation into 10^7 AX4 cells. Gene disruption in transformants was confirmed by PCR using primers located outside the cloned sequences.

To generate *grlE*-null strains by homologous recombination, a 1546 bp genomic fragment starting 178 bp before the initiation codon of GrlE was amplified by PCR and cloned in the pGEMT-EASY vector (Promega A1360). The BSR cassette from pBSR479 (Puta and Zeng, 1998) was cloned in the unique *Bam*HI restriction site of pGEMT-EASY-GrlE. For gene disruption, 10 μg of the plasmid was linearized with *Not*I before electroporation into 10^7 AX4 cells. Gene disruption in transformants was confirmed by PCR using primers located outside the cloned sequences.

The KP strain was used for the monolayer sporogenous bio-assays as previously described (Anjard et al., 1998). The level of SDF-2 was determined by testing serial dilutions in the bio-assay. The lowest effective dilution is defined as 1 unit (Anjard et al., 1998). Viability was determined by plating spores after treatment with 0.5% Triton-X100 on plates spread with bacteria. The number of plaques seen after 4 days incubation at 22°C was compared with that seen with control AX4 spores treated identically.

The efficiency of spore formation and the accumulation of SDF-2 was determined in mid-culminants collected from filters after 20 to 24 hours of development, depending on the strain. To determine the response of cells developed to mid-culmination, fruiting bodies were collected in 1 ml buffer and washed twice with 1 ml buffer. Cells (10^5) were then deposited into each of the wells of a six-well plate with 2 ml buffer with or without addition of 1 pM SDF-2, 10 nM GABA or 1 μM glutamate. After 10 minutes incubation, an aliquot of the supernatant was harvested to determine the amount of SDF-2 produced. An hour later the number of spores in each well was counted to determine the ratio of spores in the treated wells to that in the untreated wells.

The amount of SDF-2 produced in response to the various treatments was determined using the sporogenous assay. No SDF-2 (less than 2 units/10^3 cells) was detected in the supernatant of uninduced cells.

GrlE binding assay

Wild type AX4 cells and *grlE*$^-$ cells were developed on filters to the culmination stage, collected and washed three times by centrifugation in 10 ml buffer containing 20 mM MES pH 6.2, 20 mM NaCl, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$. Dissociated cells were suspended at 10^7 cells/ml in cold buffer. Cell suspension (500 μl) was incubated with 1 nM ^{3}H-CGP 54626 (American Radiolabelled Chemical, ART 715) for 1 hour on ice in the presence or absence of 10 μM CGP 55845. Cells from 400 μl of the suspension were collected on GF/C glass filters (Whatman, 1822 024) using a vacuum manifold and washed three times with 2 ml cold buffer. The amount of bound ^{3}H-CGP 54626 on the filters was measured in a liquid scintillation counter. Each experiment was carried out in triplicate.

Antibodies

The antibodies to AcbA have been previously described (Anjard and Loomis, 2005). A 1:500 dilution was added together with GABA to block SDF-2 production.

The rabbit polycolonal antibodies to the protease domain of TagC were raised to recombinant protein generated from the PCR amplified region of *tagC* that encodes the protease domain (S.-C. Chae and W.F.L., unpublished). The serum blocked processing of AcbA to SDF-2 after dilution of 1:2,000. Pre-immune serum did not block processing of AcbA to SDF-2 after dilution of 1:50. Antibodies were purified from the immune serum on a proteinA sepharose column before dialysis against phosphate-buffered saline.

Northern blot analyses

RNA was isolated from 10^8 developed cells using Trizol Reagent (Gibco BRL). Electrophoretic separations of RNA (20 μg per sample) and transfer to Nylon membranes (MagnaGraph) were as described by Shaulsky and Loomis (Shaulsky and Loomis, 1993). Probes for *grlE* were generated from the 979 bp fragment of pGEMT-EASY-GrlE isolated after digestion with *Bam*HI and *Sph*I. Random hexamers were used as primers for the Klenow fragment of DNA polymerase with 150 ng of the DNA fragment to incorporate ^{32}P dCTP.

RESULTS

Response to GABA

Addition of as little as 1 nM GABA to KP cells (Anjard et al., 1992) that had developed for 18 hours induced rapid sporulation (Fig. 1A). Lower concentrations had little effect, whereas higher concentrations did not further increase the level of sporulation. GABA might be directly inducing encapsulation or could be inducing the release of AcbA and its processing to SDF-2, which could subsequently induce encapsulation. Therefore, we added antibodies specific to AcbA simultaneously with GABA to inhibit the SDF-2 pathway. As can be seen in Fig. 1A, the antibodies completely blocked the sporulation response to GABA at all concentrations tested. These results indicate that GABA itself does not induce sporulation but results in the release of AcbA and the generation of SDF-2. As a feedback loop amplifies SDF-2 generation leading to maximal sporulation (Anjard and Loomis, 2005), the response to GABA appears to be very sharp.

The time course of appearance of SDF-2 in the supernatant was determined following addition of 10 nM GABA to developed KP cells. The level of SDF-2 in the buffer increased about 1000-fold in a two minute period starting 3 minutes after the addition of GABA (Fig. 1B). Priming of cells with SDF-2 results in a burst of SDF-2 production after a delay of only 15 seconds (Anjard et al., 1998). As we saw no increase in the level of SDF-2 for 3 minutes after the addition of GABA, the subsequent sharp increase may have resulted from a priming effect of SDF-2. As the positive feedback loop is dependent on binding of SDF-2 to its receptor, DhkA, we could determine the effect of GABA in the absence of SDF-2 priming by carrying out the experiment in KP cells in which we had disrupted

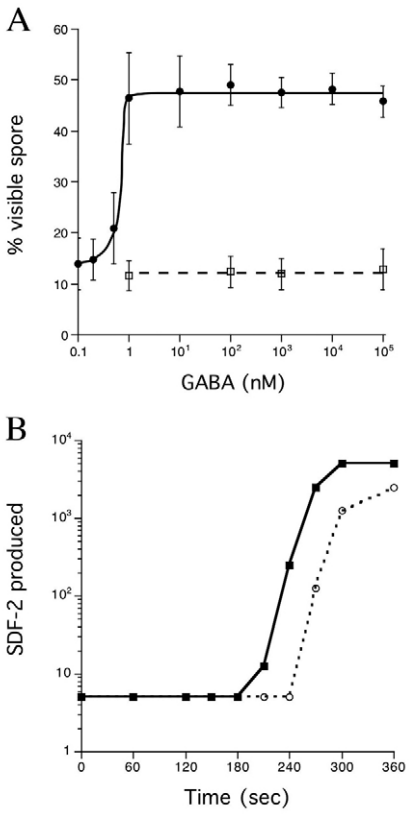

Fig. 1. GABA induction of spore formation and production of SDF-2. (**A**) KP cells developing as monolayers were treated with various concentrations of GABA and the proportion of spores determined after 1 hour (circles). They were also incubated with antibodies to AcbA for 30 seconds before adding various concentrations of GABA (squares). Each experiment was repeated three to five times; the error bars represent one s.d. (**B**) 10 nM GABA was added to KP cells (squares) and *dhkA*$^{-}$/K cells (circles) developing as monolayers. Aliquots of the surrounding buffer were collected at the times indicated and stored frozen. SDF-2 activity was determined in serial dilutions of the samples using the KP cell bio-assay as previously described (Anjard et al., 1998). The amount of SDF-2 is given as units per 10^3 cells. The lower limit in this assay is five units per 10^3 cells.

dhkA, the gene encoding the SDF-2 receptor. Although the lag following addition of GABA was extended to 4 minutes, GABA induced the generation of SDF-2 in *dhkA*$^{-}$ KP cells at the same rate and to the same high level as it did in KP cells (Fig. 1B). Thus, the positive-feedback loop of SDF-2 is not essential for the response to GABA, although it accelerates the response. Although *dhkA*$^{-}$ KP cells released SDF-2 in response to GABA, they were not induced to sporulate as these cells do not have the SDF-2 receptor (data not shown).

Exposure of TagC protease

Unprimed cells do not process AcbA to SDF-2, apparently because the protease activity of TagC is not exposed on the surface of prestalk cells until after priming (Anjard and Loomis, 2005). As the induction of sporulation by GABA is mediated by SDF-2 binding to its receptor DhkA, GABA must induce not only release of AcbA from prespore cells but also exposure of the TagC protease domain such that AcbA can be cleaved to form the signalling peptide. Addition of antibodies raised to the protease domain of TagC simultaneously with either GABA or SDF-2 blocked SDF-2 production (Table 1). To demonstrate that the epitope is not present on the surface before priming, we treated unprimed cells with the anti-TagC antibodies and then washed the cells extensively before priming. The cells responded either to GABA or to SDF-2 by rapidly processing recombinant AcbA into SDF-2 (Table 2). However, if the cells had been primed with either GABA or SDF-2 before addition of anti-TagC antibodies, they processed less than 1% as much recombinant AcbA into SDF-2 as cells that were not treated with antibodies (Table 2). It appears that the antibody is able to recognize the protease domain of TagC on the surface of cells treated with GABA or SDF-2 and continues to block the ability to processes AcbA, even when free antibody is removed. However, the epitope is not present on the surface of cells prior to treatment with either GABA or SDF-2, and the antibody has no effect when removed before addition of GABA or SDF-2.

Table 1. Inhibition of SDF-2 production upon addition of anti-TagC antibodies or ABC inhibitors

Additions	SDF-2 production
None	No
0.1 pM SDF-2	Yes
0.1 pM SDF-2 + anti TagC	No
10 nM GABA + anti TagC	No
0.1 pM SDF-2 + 2 mM vanadate	No
10 nM GABA + 2 mM vanadate	No
0.1 pM SDF-2 + 100 μM verapamil	No
10 nM GABA + 100 μM verapamil	No
0.1 pM SDF-2 + 200 μM corticosterone	No
10 nM GABA + 200 μM corticosterone	No

Developed KP cells were treated with the various compounds and the supernatants collected after 1 hour, serially diluted and added to test KP cells.
'Yes' indicates at least 5000 units of SDF-2 per 10^3 cells; 'No' indicates fewer than 10 units of SDF-2 per 10^3 cells.
All experiments were repeated at least three times.

The protease domain of TagC is fused with an ABC domain, which is expected to be embedded in the membrane and act as a transporter (Shaulsky et al., 1995). ABC transporters have cytoplasmic ATP-binding sites and couple hydrolysis of ATP to export of a variety of compounds. The protease domain of TagC is predicted to be cytoplasmic but may use the attached transporter domain for its exposure on the surface. In support of this model, we found that inhibition of ATPase activity by vanadate before priming with GABA or SDF-2 blocked production of SDF-2 (Table 1). We also found that 100 μM verapamil and 100 μM corticosterone, known inhibitors of ABC proteins (Ambudkar and Gottesman, 1998; Good and Kuspa, 2000), blocked SDF-2 production in response to either GABA or SDF-2 (Table 1). These results are consistent with the ABC domain of TagC being responsible for exposing the protease domain so that it can convert AcbA to SDF-2.

GrlE is the GABA receptor

The product of the *grlE* gene (DDB0231976) is predicted to be a G-protein-coupled seven transmembrane receptor of family 3 GPCRs (Hereld, 2005). There is a signal sequence for membrane insertion at the N terminus that is followed by a ligand-binding domain with homology to $GABA_B$ and glutamate metabotropic receptor domains. The seven-transmembrane region is towards the C terminus. $GABA_B$ receptors and glutamate metabotropic receptors can be distinguished on the basis of specific amino acids in their binding domains. The GrlE ligand-binding domain shows higher similarity to the $GABA_B$ receptors than to glutamate metabotropic receptors.

Table 2. Exposure of the protease domain of TagC

Treatment before addition of antibodies	Treatment after removal of antibodies	SDF-2 production from recombinant AcbA (U/10^3 cells)
Unprimed	None	<10
Unprimed	Primed	12,000±1000
Primed	None	70±30
Primed	Primed	30±20

Unprimed cells were treated with antibodies to the protease domain of TagC for 5 minutes and then extensively washed before being primed with either 10 nM GABA or 10 pM SDF-2. Primed cells were given either 10 nM GABA or 10 pM SDF-2 for 5 minutes, and then washed before adding antibodies to the protease domain of TagC. After 5 minutes, free antibody was washed away and half of the cells were primed with either GABA or SDF-2. Five minutes after priming, the cells were washed and incubated with 10 pmoles recombinant AcbA for 30 minutes before the level of SDF-2 was determined in the supernatant. All experiments were repeated at least three times.

However, the difference in similarities is small and so its ligand specificity cannot be predicted with confidence. Glutamate metabotropic receptors in animals have a cysteine-rich domain that is necessary for forming stable dimers (Kunishima et al., 2000). This region is missing in the $GABA_B$ receptors of animals, as well as in GrlE. We used homologous recombination with a construct in which the blasticidin resistance gene used for selection was inserted near the start of *grlE*. We isolated two independent strains in which the size of diagnostic PCR products indicated that the endogenous gene was replaced by the disrupted copy (data not shown). These strains grew well and proceeded through morphogenesis normally. However, they both produced less than a third as many viable spores as did wild-type strains. No SDF-2 activity could be recovered from the fruiting bodies of these strains. One of these mutants was chosen for further study.

Cells were dissociated from wild-type and *grlE*-culminants and incubated in buffer in multitest wells. 10 nM GABA or 1 pM SDF-2 was added and the number of spores counted microscopically 1 hour later. The number of spores in the wild type strain AX4 was induced over threefold by either GABA or SDF-2 relative to the level seen in the absence of inducers (Fig. 2). However, there was no significant induction of sporulation by GABA in the *grlE*$^-$ cells.

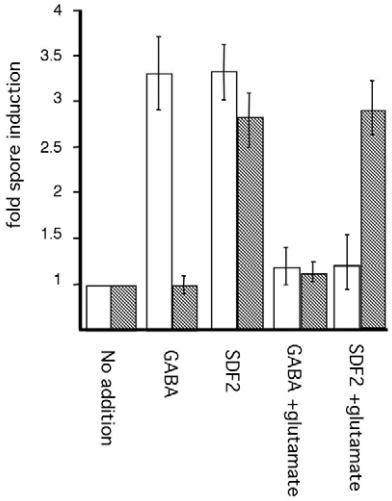

Fig. 2. Spore formation in wild type and *grlE*$^-$ strains. Early culminants of the wild-type strain AX4 (white columns) and the *grlE*$^-$ null strain (hatched columns) were disaggregated, washed and deposited at 10^5 cells per well in 2 ml buffer. GABA (100 nM); 10 pM SDF2; 100 nM GABA and 10 μM glutamate; or 10 pM SDF-2 and 10 μM glutamate were then added to the wells as indicated. The proportion of spores was determined 1 hour later and compared with cells that received no additions. Each experiment was repeated three to five times; the error bars represent 1 s.d.

However, these cells responded to SDF-2 normally (Fig. 2). It appears that cells lacking GrlE do not respond to GABA by generating SDF-2, although they respond to SDF-2 when it is added exogenously.

As GrlE is almost as closely related to metabotropic glutamate receptors as it is to $GABA_B$ receptors, we determined whether glutamate would induce rapid sporulation or affect induction by GABA. We found that glutamate alone had no inducing effect even when added up to 1 μM (data not shown). However, it acted as a competitive inhibitor of GABA for the induction of encapsulation (Fig. 2, Fig. 3A). Glutamate could completely inhibit the induction of sporulation by GABA if present at a 100 times higher concentration. This was somewhat surprising because the metabotropic receptors in mammals are completely specific for either GABA or glutamate, and show no evidence for cross competition. Even more surprisingly, we found that glutamate inhibited the ability of SDF-2 to induce rapid sporulation in a non-competitive manner (Fig. 2, Fig. 3B). Maximal inhibition was observed at 100 nM glutamate, independent of the amount of SDF-2 that was present. It appears that both GABA and glutamate can bind to GrlE but that they each elicit different responses. Induction of sporulation by SDF-2 was unaffected by 10 μM glutamate in *grlE*$^-$ null mutants further indicating that glutamate is acting through the GrlE receptor (Fig. 2).

To determine the specificity of the response to glutamate and GABA, we tested aspartate, arginine, glutamine, methionine or serine to see if they would either induce rapid sporulation or inhibit induction by GABA or SDF-2. Even when added at 5 mM these compounds had no significant effects on spontaneous sporulation or the response to either GABA or SDF-2 (data not shown).

To confirm that GrlE is the GABA receptor, we used the high-affinity GABA antagonists CPG 54626 and CGP 55845, which have been shown to be specific for $GABA_B$ receptors (Davies et al., 1993). The affinity of mammalian GABA-B receptors for these compounds is tenfold higher than it is for GABA facilitating binding assays. We incubated 1 nM ^{3}H-CGP 54626 with 5×10^6 cells dissociated from culminants of either the wild-type strain or the *grlE*$^-$ mutant strain for an hour at 0°C and then collected them on filters. Non-specific binding was measured by adding 10,000-fold excess unlabelled CGP55845 (Table 3). We found that wild-type cells would specifically bind 110 fmoles CGP4626, while *grlE*$^-$ mutant cells bound only 15 fmoles, which is within the error margin of the assay.

Expression of grlE

Northern analyses of RNA collected at 2 hour intervals throughout development showed that *grlE* mRNA is very low in vegetative cells but starts to accumulate in the first 2 hours after the initiation of development (Fig. 4). It reaches a peak at 4 hours and then declines. However, a slightly larger *grlE* mRNA accumulates after 10 hours

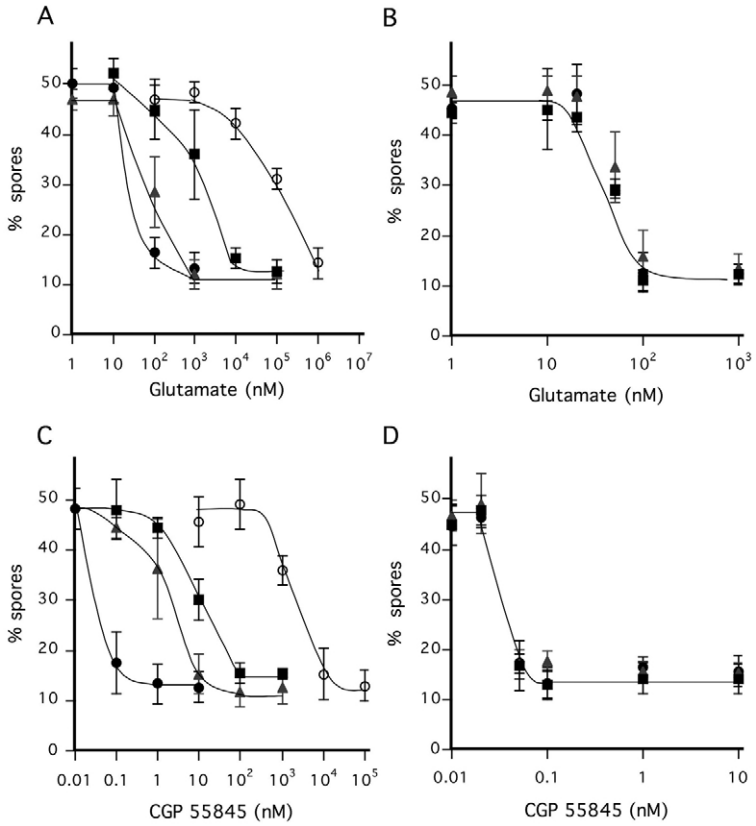

Fig. 3. Inhibition of spore induction. (**A**) KP cells developing as monolayers were treated with various concentrations of GABA and glutamate, and the proportion of spores determined after 1 hour: 1 nM GABA (black circles), 10 nM GABA (triangles), 100 nM GABA (squares), 1 μM GABA (white circles). Glutamate was added simultaneously with GABA at the concentrations indicated. (**B**) SDF-2 was added to the cells at 0.1 pM (circles); 10 pM SDF-2 (triangles); 10 nM (squares). Glutamate was added simultaneously at the concentrations indicated. (**C**) GABA and CGP 55845 were added at various concentrations and the proportion of spores determined after 1 hour; 1 nM GABA (black circles), 10 nM GABA (triangles), 100 nM GABA (squares), 1 μM GABA (white circles). CGP 55845 was added simultaneously at the concentrations indicated. (**D**) SDF-2 was added to the cells at 0.1 pM (circles); 10 pM SDF-2 (triangles); 10 nM (squares). CGP 55845 was added simultaneously at the concentrations indicated.

and remains until 22 hours of development. As the gene is expressed several hours before the initial cell type divergence and maintained until culmination, it is likely that GrlE is present in both prespore and prestalk cells, where it can mediate both release of AcbA from prespore and exposure of the TagC protease domain on prestalk cells. As expected, no signal was observed on RNA samples prepared from developing *grlE*-null cells, confirming that the insertion disrupted this gene (Fig. 4).

GABA signal transduction pathway

Interest in the metabotropic receptors in brain function has resulted in a large number of well characterized pharmacological reagents directed at these receptors. We tested several compounds for the ability to block induction of rapid sporulation of KP cells by GABA or SDF-2. We found that CPG 55845 is a competitive inhibitor of GABA induction of sporulation in the nanomolar range (Fig. 3C). The relative affinity of the receptor for CGP55845 appears to be 10-fold higher than for GABA, while its relative affinity for glutamate is 100-fold lower than for GABA (Fig. 3A). Therefore, we would expect it to block SDF-2 induction of sporulation at lower concentrations than glutamate. As can be seen in Fig. 3D, CGP55845 blocks the induction of sporulation by 10 pM SDF-2 when present at 0.05 nM or higher concentrations. As the apparent affinity for this compound is similar to that found for mammalian receptors, the structure of the ligand-binding domains of these GPCRs must be quite similar.

To follow the signal transduction pathway further, we tested several other compounds known to inhibit specifically common downstream components. We found that both CGP55845 and CGP54626, competitive inhibitors of $GABA_B$ receptors, blocked the generation of SDF-2 in response to either SDF-2 or GABA (Table

Table 3. Binding of ^{3}H-CGP 54626 to whole cells

Strain	Total binding	Non-specific binding	Specific binding
Ax4	213±13	112±17	110±30
grlE$^-$	58±5	43±2	15±7

Binding is expressed as fmol ^{3}H-CGP 54626 bound per 10^8 cells. Non-specific binding was determined by addition of 10,000-fold excess CGP55845. Results are averages of triplicate determinations.

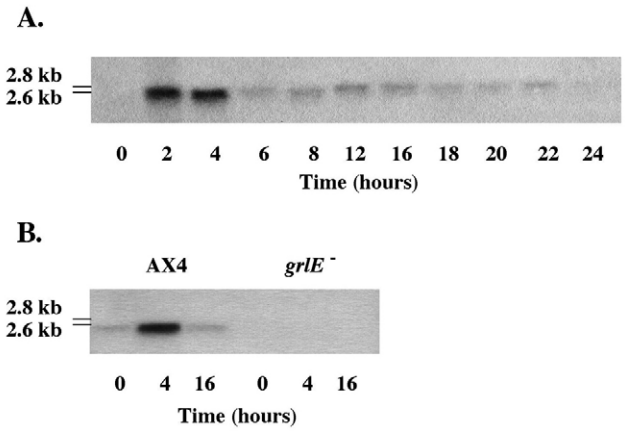

Fig. 4. Time course of GrlE expression. (**A**) Total RNA was extracted from wild-type AX4 cells harvested at the indicated time of development and electrophoretically separated on a 1.2% agarose gel before being transferred to a nylon membrane. The membrane was hybridized with a probe for *grlE* (see Materials and methods). (**B**) Samples were collected from wild-type and *grlE*$^-$ null cells that had developed for 0, 4 and 16 hours, and probed for *grlE* mRNA. Consistent loading in each lane was confirmed by staining for rRNA.

Table 4. Comparison of drugs and mutant strains

A Drugs

Drug	Concentration (μM)	SDF-2 production upon SDF-2 priming	SDF-2 production upon GABA priming	SDF-2 production in the presence of glutamate
None		Yes	Yes	No
CGP 55845	0.001	No	No	NA
CGP54626	0.001	No	No	NA
LY294002	1	Yes	No	No
Akt inhibitor IV	1	Yes	No	No
Akt inhibitor VIII	10	Yes	Yes	No
H-89	10	No	Yes	No

B Strains

Strain	SDF-2 in sorus	SDF-2 production upon SDF-2 priming	SDF-2 production upon GABA priming	SDF-2 production in presence of glutamate
AX4	Yes	Yes	Yes	No
gadA$^-$	No	Yes	Yes	No
grlE$^-$	No	Yes	No	Yes*
acbA$^-$	No	No	No	NA
pik1$^-$*2*$^-$	No	Yes	No	No
pkbR1$^-$	No	Yes	No	No
acaA$^-$*K*	Yes	Yes	Yes	No
gα 9$^-$	Yes	Yes	Yes	Yes

'Yes' indicates at least 5000 units of SDF-2 per 10^3 cells; 'No' indicates fewer than 10 units of SDF-2 per 10^3 cells. The minimum effective concentration of the compounds on KP cells is given. Cells of the various strains were dissociated from culminants and treated with 1 pM SDF-2 or 10 nM GABA. *pik1*$^-$ *2*$^-$ and *acaA*$^-$ K cells can develop if plated at high density such that they can form aggregates by accretion. To determine whether glutamate inhibited SDF-2 production in cells treated with the various drugs or in the mutant strains, 1 μM glutamate was added simultaneously with SDF-2 or GABA, and the production of SDF-2 was measured in the supernatant after 30 minutes. All experiments were repeated three times. NA, not applicable.
*With SDF-2 priming but not with GABA priming.

4). LY294002, an inhibitor of PI3 kinases, as well as AKT inhibitor IV, an inhibitor of the PKB-related protein kinases, blocked induction of SDF-2 production by GABA but had no effect on induction by SDF-2 itself (Table 4). Thus, it is likely that PI3K and PKB-R1 act downstream of GrlE. We also tested AKT inhibitor VIII, which is targeted to the PH domain of AKT, and H89 which inhibits PKA. Neither of these compounds had any measureable effect, suggesting that the GABA signal transduction does not involve the classical AKT or PKA. Addition of 1 μM glutamate inhibited SDF-2 production in response to either GABA or SDF-2, even in the presence of LY294002 or the Akt inhibitors (Table 4). Although these results are not surprising for cells primed with GABA, as glutamate is a competitive inhibitor of GABA, they indicate that the pathway by which glutamate inhibits SDF-2 priming is independent of PI3 kinases or PKB-R1.

To further explore the signal transduction pathway we determined whether mutants lacking the various components accumulated SDF-2 in fruiting bodies (Table 4). Unlike wild-type fruiting bodies which contain high levels of SDF-2, those made by the *gadA*$^-$ mutant have no measurable SDF-2. It appears that the late glutamate decarboxylase is essential for normal generation of SDF-2 during development. If either SDF-2 or GABA were added to *gadA*$^-$ cells dissociated from culminants, they rapidly generated SDF-2, showing that they had developed to the stage where they can respond to the sporulation signals. However, if glutamate was present at 100 times the level of GABA, there was no production of SDF-2 in response to GABA (Table 4).

There is no measurable SDF-2 in fruiting bodies of *grlE*$^-$ cells, indicating that the GABA receptor is necessary for SDF-2 production during development. SDF-2 is not generated by cells dissociated from *grlE*$^-$ culminants after treatment with GABA, although they respond normally to added SDF-2. As shown in Fig. 2, glutamate does not block induction of sporulation by SDF-2 in this strain.

As expected, *acbA*$^-$ cells, which cannot make the precursor of SDF-2, do not have measurable SDF-2 in their fruiting bodies and do not generate SDF-2 upon priming by either SDF-2 or GABA (Table 3). However, they can generate SDF-2 if provided with recombinant AcbA, as long as they are primed by GABA or SDF-2 (data not shown). The *pik1*$^-$ *2*$^-$ and *pkbR1*$^-$ mutants also do not contain SDF-2 in their fruiting bodies, although they can respond to added SDF-2 by producing high levels of SDF-2. GABA does not induce SDF-2 generation in these strains (Table 3). As expected from the lack of inhibition by H89, cells lacking the G protein-coupled adenylyl cyclase, AcaA, that overexpress *pkaC* responded normally to GABA and SDF-2 (Table 4).

Glutamate inhibited SDF-2 production in response to either GABA or SDF-2 in each of the strains except the one lacking the GABA/glutamate receptor GrlE (Table 4). This strain responds to SDF-2 but not to GABA by production of SDF-2 (Table 4). These results confirm that glutamate inhibits SDF-2 production in response to SDF-2 by a pathway that is independent of either PI3 kinase or PKB-R1. The observation that glutamate did not inhibit SDF-2 production in response to SDF-2 in the strain lacking Gα9, while GABA still induced SDF-2 in this strain (Table 4), further indicates that the signal transduction pathways for GABA and glutamate are separate although both require the GrlE receptor.

DISCUSSION

We disrupted the prespore gene encoding glutamate decarboxylase, *gadA*, as part of a project to determine the roles of prespore-specific genes. When we observed that the efficiency of sporulation was decreased and that half of the spores were permeable to propidium iodide, we considered the possibility that GABA might be directly involved in spore induction. We had previously found that mutations in the genes of the SDF-2 pathway, including *acbA*, *tagC* and *dhkA*, result in reduced sporulation and the production of propidium iodide permeable spores, a sign of compromised spores (Wang et al., 1999;

Anjard and Loomis, 2005). The discovery that 1 nM GABA induces rapid encapsulation of competent *Dictyostelium* cells demonstrates that this neurotransmitter has an ancient heritage as an intercellular signal. GABA does not directly induce sporulation, but results in the rapid production of SDF-2 which then induces encapsulation when it binds to its receptor histidine kinase DhkA. If processing of AcbA to SDF-2 is blocked by the simultaneous addition of antibodies to AcbA, there is no increase in the number of spores following addition of GABA. However, the rapid release of SDF-2 following addition of GABA is not dependent on DhkA, ruling out a direct effect of GABA on this receptor.

Production of GABA by glutamate decarboxylase late in development appears to be essential for accumulation of SDF-2 as fruiting bodies of *gadA*$^-$ strains are devoid of SDF-2 (Table 4). The requirement for GABA signaling to initiate SDF-2 production is further supported by our observation that SDF-2 is also missing from the fruiting bodies of *grlE*$^-$ mutant strains that lack the GABA receptor. The fact that these strains are able to complete terminal differentiation and make spores at all is probably due to the other peptide signal, SDF-1, which also induces encapsulation (Anjard et al., 1997). Fruiting bodies of *gadA*$^-$ and *grlE*$^-$ mutants that lack SDF-2 were shown to have normal levels of SDF-1 (data not shown).

The GABA receptor, GrlE, is a seven transmembrane G-protein-coupled receptor in which the ligand-binding domain is similar to those of the $GABA_B$ family but also shows some similarity to the ligand-binding domains of metabotropic glutamate receptors. In fact, both GABA and glutamate appear to bind to GrlE. Glutamate acts as a competitive inhibitor of GABA and can block the ability of GABA to induce rapid encapsulation if present at 100-fold higher concentrations than GABA. Moreover, 100 nM glutamate blocks the induction of sporulation by SDF-2. This inhibitory response is missing in *grlE*-null cells showing that it is mediated through the GrlE receptor (Fig. 2). The GrlE receptor is not necessary for the cells to respond to SDF-2 by rapid encapsulation indicating that it does not directly interact with the SDF-2 receptor DhkA. The $GABA_B$ specific inhibitors CGP55845 and CGP5426 are effective inhibitors of GrlE (Table 4), indicating that the binding site is similar to the $GABA_B$-binding site.

Signal transduction pathways from GPCRs often result in activation of adenylyl cyclase, such that PKA activity increases. Although the PKA inhibitor H-89 blocked the production of SDF-2 in response to SDF-2 priming as previously reported (Anjard et al., 1998), it did not inhibit production of SDF-2 in response to GABA (Table 3). Although PKA plays a myriad of roles in development of *Dictyostelium,* it does not seem to be involved in signal transduction from GrlE. Moreover, we found that cells lacking adenylyl cyclase but overexpressing PKA (*acaA*$^-$ K) respond to both GABA and SDF-2 by rapidly producing high levels of SDF-2 (Table 4). The fact that they respond to GABA normally indicates that activation of adenylyl cyclase is not necessary for the signal transduction pathway leading to SDF-2.

Some GPCRs are coupled to phospholipase C and use diacylglycerol and IP_3 as a second messengers (van Dijken et al., 1997; Rhee, 2001). We treated the cells with 6 μM U73122, a drug that has been shown to block phospholipase C in *Dictyostelium* (Lyden and Cotter, 1995; Seastone et al., 1999), and found that it had no effect on the ability of GABA to induce rapid production of SDF-2 (data not shown). It appears that PLC is not part of the GrlE signal transduction pathway.

PI3kinase and Akt/PKB are also frequently found in signal transduction pathways downstream of GPCRs (Meili et al., 1999; Meili et al., 2000; Manahan et al., 2004; Sasaki et al., 2004). We tested the PI3 kinase inhibitor LY294002 and Akt inhibitor IV and found that addition of either drug at 1 μM completely blocked the response to GABA that results in generation of SDF-2. Akt inhibitor IV is targeted to the kinase which activates PKB and PKB-related proteins. However, Akt inhibitor VIII, which is targeted to the PH domain of Akt, had no observable effect. The post-aggregation PKB-related kinase, PkbR1, unlike the aggregation stage Akt1, does not carry a PH domain (Meili et al., 2000). Therefore, we determined whether a strain lacking PKB-R1 would respond to SDF-2 priming or GABA priming. We found that the *pkbR1*-null strain failed to produce SDF-2 when primed by GABA, although it responded normally to SDF-2. As expected, a strain in which the two major PI3 kinase genes, *pik1* and *pik2*, were disrupted failed to respond to GABA. Neither the *pik1*$^-$ *pik2*$^-$ double mutant nor the *pkbR1*-null strain accumulated any measurable SDF-2 in their fruiting bodies, once again showing that GABA signaling is essential for SDF-2 production (Table 4). Although we cannot rule out that PI3kinase and PKB R1 might function in parallel to induce AcbA release, it has previously been shown that these enzymes act in a linear pathway at an earlier stage of development in *Dictyostelium* (Meili et al., 1999; Meili et al., 2000; Manahan et al., 2004; Sasaki et al., 2004). Therefore, we suggest that when GABA binds to GrlE, PI3 kinases are activated leading indirectly to activation of PKB-R1 and triggering release of AcbA from prespore cells (Fig. 5).

Once AcbA is released, it is rapidly processed into SDF-2 in a process that is dependent on the prestalk specific ABC-protease TagC. In the absence of priming, there is no processing of recombinant AcbA into SDF-2. Cells in which the *tagC* gene is disrupted are unable to process recombinant AcbA into SDF-2. Likewise, primed cells treated with antibodies to the protease domain of TagC are unable to process AcbA into SDF-2, even when

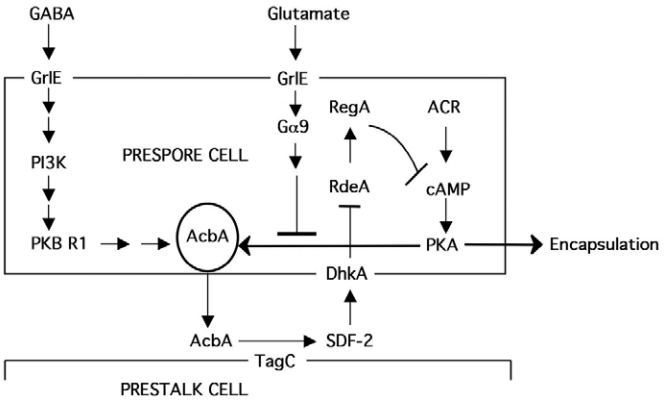

Fig. 5. Model of signal transduction pathways during terminal differentiation. When GABA is bound to GrlE on the surface of prespore cells, the signal is transduced via PI3 kinase and PkbR1 to result in release of AcbA, the precursor of SDF-2. The same pathway in prestalk cells results in the exposure of the protease domain of TagC, such that it can process AcbA into SDF-2. When glutamate is bound to GrlE, a distinct signal transduction pathway results in the inhibition of release of AcbA in response to SDF-2. When SDF-2 binds its receptor DhkA, phospho-relay to RdeA is inhibited and may be reversed, resulting in a decrease in the activity of the internal cAMP phosphodiesterase RegA. The subsequent increase in cAMP generated by the adenylyl cyclase ACR activates PKA which triggers release of AcbA, as well as rapid encapsulation of prespore cells. Inhibition of phosphorelay to RegA in prestalk cells results in exposure of the protease domain of TagC.

free antibody is washed away (Table 2). However, the TagC protease epitope is not exposed before priming, as shown by the fact that antibodies to it do not block the activity if they are washed out before priming and the cells presented with recombinant AcbA. It appears that signaling by either GABA or SDF-2 results in the presentation of the TagC protease on the surface where it can process AcbA. The protease domain is followed by an ABC domain in TagC and inhibitors of the ABC transport function block exposure of the protease domain (Table 1). The six-transmembrane ABC domain may be directly involved in the presentation of the attached protease domain on the cell surface.

It was surprising to find that glutamate not only inhibited GABA induction of encapsulation but also inhibited the response to SDF-2 (Fig. 2). These sporulation signals use very different signal transduction pathways yet seem to interact to regulate release of AcbA. Although both GABA and glutamate work through the GrlE receptor, they elicit opposite effects. Moreover, the glutamate pathway is not sensitive to LY294002 or Akt inhibitor IV, whereas the GABA pathway is blocked by these drugs. The absence of inhibition by glutamate of SDF-2 production in response to SDF-2 in a strain lacking Gα9 indicates that GrlE is coupled to a trimeric G protein with this specific subunit. However, GABA induced SDF-2 production normally in the strain lacking Gα9 showing that GrlE is not obligatorily coupled to this G protein. It appears that GrlE interacts with two different trimeric G proteins, depending on which ligand is bound, and that one stimulates the PI3 kinase pathway while the other does not. To account for the fact that glutamate can inhibit the ability of the SDF-2 to act through its receptor histidine kinase DhkA and cause release of AcbA, we would have to consider a separate inhibitory pathway that functions when glutamate binds to GrlE (Fig. 5).

$GABA_B$ receptors in mammalian cells are heterodimers formed from two highly related transmembrane proteins, only one of which binds GABA (Kniazeff et al., 2002). The other subunit is responsible for transducing the signal within the cell. However, mammalian metabotropic glutamate receptors function as homodimers (Kniazeff et al., 2004; Tateyama et al., 2004). GrlE may function as a monomer, a homodimer or a heterodimer in *Dictyostelium*. There are many potential partners for GrlE; the *Dictyostelium* genome encodes 14 other family 3 GPCRs (Eichinger et al., 2005; Hereld, 2005). However, none of them have regions similar to the ligand binding domain of GrlE. Nevertheless, if they work in the same way as the GB2 subunit of mammalian $GABA_B$ receptors, they would not be expected to bind GABA. Systematic study of these genes may uncover an interacting partner.

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