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Trace Amines Depress $Gaba_b$ Response In Dopaminergic Neurons By Inhibiting Girk Channels

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Running title: Trace amines modulate $GABA_B$ response.

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ABBREVIATIONS: TA, trace amine; β-PEA, β-phenylethylamine; TYR, tyramine; GIRK , G-βγ gated inwardly rectifying potassium; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; DA, dopamine; ACSF, artificial cerebrospinal fluid; IPSP, inhibitory post synaptic potential; EPSP, excitatory post synaptic potential; IBac, baclofen activated outward current; DAT, dopamine transporter.

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ABSTRACT

Trace amines (TAs) are present in the central nervous system where they upregulate catecholamine release and are implicated in the pathogenesis of addiction, attention deficit hyperactive disorder, Parkinson's disease and schizophrenia. By using intracellular and patch-clamp recordings from dopaminergic cells in the rat midbrain slices, we report a depressant postsynaptic action of two TAs, β-phenylethylamine (β-PEA) and tyramine (TYR) on the $GABA_B$-mediated slow inhibitory postsynaptic potential (IPSP) and baclofen-activated outward currents. β-PEA and TYR activated G-proteins interfering with the coupling between $GABA_B$ receptors and G-βγ gated inwardly rectifying potassium (GIRK) channels. This is the first demonstration that β-PEA and TYR depress inhibitory synaptic potentials in neurons of the central nervous system supporting their emerging role as neuromodulators.

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Introduction

Trace amines are biological compounds previously thought to have a marginal role in regulating neuronal functions. They are present at low levels in mammalian neuronal tissue and are packaged and released along with traditional amines (Boulton, 1976; Boulton, 1982; Durden and Philips, 1980; Parker and Cubeddu, 1988). They have been considered as "false transmitters", which displace active biogenic amines from their stores and act on catecholamine transporters in an amphetamine-like manner (Janssen et al., 1999; Mundorf et al., 1999.). Recently, a specific role for TAs has been suggested by the discovery of two subtypes of G protein-coupled TA receptors, TA_1 and TA_2, both functionally coupled to increases in cAMP formation (Borowsky et al., 2001; Bunzow et al., 2001) presumably via the $G\alpha_s$ class of G-proteins. However, the physiological actions of these receptors are poorly understood and research efforts have so far been confined to reconstituted *in vitro* expression systems. Considering that mRNA for the TA_1 and TA_2 receptors is present in the substantia nigra (SN)/ventral tegmental area (VTA) (Borowsky et al., 2001) and cyclic AMP-dependent protein kinase A (PKA) facilitates $GABA_B$ currents (Bonci and Williams, 1996; Cameron and Williams, 1993; Couve et al., 2002), we postulated that the biogenic amines, β-PEA and TYR, could increase the evoked $GABA_B$-slow inhibitory post synaptic potential (IPSP) in midbrain dopaminergic neurons. Unexpectedly, our electrophysiological experiments demonstrated that β-PEA and TYR, reduce the $GABA_B$ IPSP by a G-protein coupled postsynaptic mechanism. This depressant effect was mediated by neither PKA or protein kinase C (PKC)-dependent phosphorylation nor by phospholipase C (PLC) activation, but results mainly from TA-activated G-proteins interfering with the opening of $GABA_B$-gated GIRK channels. Together, our results reveal a new role of β-PEA and TYR in depressing $GABA_B$ synaptic transmission. Given the fact that this phenomenon occurs in dopaminergic cells, it is relevant to the control of brain operations linked to movement, cognition and motivation.

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Materials and Methods

Slice preparation. Adult male Wistar rats (150-250 g) were used for all experiments. Standard procedures were used to obtain slices of the ventral midbrain for electrophysiological recordings (Mercuri et al., 1995). All experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Briefly the brain was dissected, mounted on an agar block and horizontal slices (250 μm) were cut by a vibratome at 8 - 10 °C. Slices were maintained in artificial cerebrospinal fluid (ACSF) for 45 min at 33 °C in an interface chamber before being transferred to a superfusing recording chamber. The ACSF contained (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, Glucose 10 and $NaHCO_3$ 19, (pH 7.4, equilibrated with a mixture of O_2 95 %/CO_2 5 %.).

Intracellular recordings. Intracellular recordings from midbrain substantia nigra pars compacta (SNc) and VTA dopaminergic neurons were performed at 35 °C in a recording chamber in which the slice was immobilized with two titanium grids and perfused at a rate of 2.5 - 3 ml/min, with ACSF solution. Neurons were identified as dopaminergic by their electrical properties that included the presence of a regular spontaneous firing activity, a relaxation of the hyperpolarizing electrotonic potentials mediated by the activation of I_h and the $GABA_B$ IPSP (Grace and Onn, 1989; Johnson and North, 1992; Mercuri et al, 1995). As no differences were observed between cells of the VTA and SNc, the data were pooled. To prevent spontaneous spikes, the membrane potential was adjusted between - 65 and - 70 mV by hyperpolarizing current injection. The recording electrodes were filled with 2M KCl and had a tip resistance of 30-80 MΩ. Synaptic events were evoked with bipolar tungsten stimulating electrodes located within the slice and having a tip separation of 300 - 600 μm. The $GABA_B$ IPSP was evoked by a train of 4-8 stimuli of 70 μs at 8 - 20 V, delivered at 70 Hz every 30 s (Johnson et al., 1992). The amplitude of synaptic potentials was measured from traces

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representing the average of four responses. All $GABA_B$ synaptic potentials were recorded in the presence of a pharmacological cocktail containing bicuculline methiodide (50 μM), strychnine (1 μM), prazosin (300 nM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), 2-amino-5-phosphonopentanoic acid (AP-5, 50 μM), SCH 23390 (3 - 10 μM) and sulpiride (1 - 3 μM) to block $GABA_A$, glycine, α_1-adrenergic, AMPA, NMDA, D_1 and D_2-like receptors respectively. In some experiments the $GABA_B$ receptor antagonist CGP 55845 (300 nM) was perfused to block the $GABA_B$ IPSP. The $GABA_A$ IPSP was evoked by a single electrical stimulus with the same cocktail of drugs used to evoke the $GABA_B$ potential but omitting bicuculline. The fast excitatory post synaptic potential (EPSP) was obtained by a single stimulus with the same cocktail of drugs used to evoke the $GABA_B$ potential but omitting CNQX and AP-5.

Patch-clamp recordings. Whole cell patch-clamp recordings were obtained with an amplifier (Axopatch 200B, Axon Instruments) from visually and electrophysiologically identified dopamine (DA) neurons in the SNc and VTA, using patch pipettes (3 - 4 MΩ) made from borosilicate glass 1.5 mm (WPI, Sarasota, FL) and pulled with a PP 83 Narishige puller (Tokyo, Japan). The electrophysiological characteristics were already published (Liss et al., 1999; Tozzi et al., 2003). Membrane currents were digitized at 5 kHz through a Digidata 1200B A/D converter, acquired and analyzed using pClamp software (Axon Instruments, Union City, CA). Pipettes were filled with a standard internal solution containing (in mM): K-gluconate 145; $CaCl_2$, 0.1; $MgCl_2$, 2; HEPES, 10; EGTA, 0.75; Mg_2-ATP, 2; Na_3GTP, 0.3; or K^+ methylsulfate, 145; KCl, 8; HEPES, 10; Mg-ATP, 2; Na_3GTP, 0.3 (pH 7.35 with KOH). The extracellular perfusate consisted of ACSF plus TTX (1 μM), sulpiride (1-3 μM) and SCH 23390 (3-10 μM). Access resistance was monitored at regular intervals. A 10 mV hyperpolarizing step (250 ms) was applied to measure membrane resistance. The current-voltage relationship was measured with a step protocol (1 second per sweep, 4 sweeps, - 60 to - 120

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mV) executed after the GIRK conductance was completely activated (GTP-γ-S + baclofen) and in the presence of the trace amine, at steady-state. All membrane potentials were corrected for the calculated liquid junction potential (13 to 15 mV).

Ca^{2+} imaging. The fluorescent ion indicator fura-2 was loaded into the cell via the patch pipette, and the fluorescence emitted at 340-380 nm excitation wavelengths was detected by a CCD camera (Photonic Science, Millham, UK) at 6 second intervals and then stored. Fluorescence values from selected regions of the neuron that included the cell body were then analyzed offline in term of ratio value changes (R).

Drugs. β-Phenylethylamine, p-tyramine, phentolamine, reserpine, dopamine hydrochloride, AP-5, bicuculline methiodide, staurosporine, Rp-cAMPS, SQ22,536 were purchased from Sigma (Milan, Italy); baclofen, CNQX, prazosin, sulpiride, SCH 23390, carbidopa, U73122 and DHPG from Tocris Cookson (Bristol, UK); tetrodotoxin from Alomone Laboratories (Jerusalem, Israel), CGP 55845 was a kind gift from Novartis; phosphatidylinositol-3,4-biphosphate ($PtIns(4,5)P_2$) was purchased from Calbiochem (La Jolla, CA); fura-2 from Molecular Probes (Leiden, The Netherlands). All drugs were bath-applied at known concentrations by switching the perfusion using a three-way tap. Baclofen or DHPG (100 μM) was applied in extracellular solution through a glass pipette whose open end (approximately 2 μm in diameter) was positioned 20-30 μm from the cell body of the patched neuron. The local application of baclofen or DHPG was performed at 20 psi with a pneumatic pico-pump (Picospritzer, WPI, Sarasota, FL, USA). PtIns(4,5)P2 was dissolved in pipette solution at a nominal concentration of 500 μM. The solution was sonicated intermittently on ice for 30 min. Sonication was repeated each time before filling a new pipette. PtIns(4,5)P2 solutions were used for 1 day only. Also the PKA inhibitor c-AMPS-Rp,triethylammonium salt was applied by intracellular dialysis. A series of

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rats were treated with reserpine 5 - 8 mg/Kg injected 15 – 24 hours before the electrophysiological experiments. These dosages produced profound hypokinesias and ptosis.

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Results

β-PEA and TYR reduce the $GABA_B$ IPSP. Using intracellular recordings with sharp microelectrodes, we studied the effects of β-PEA and TYR on the slow inhibitory transmission mediated by $GABA_B$ receptors on dopaminergic cells (n = 22). Bath application of β-PEA and TYR reduced the amplitude of the $GABA_B$ IPSP (Fig. 1a - b). This depressant action of β-PEA and TYR was observed in 19 out of 22 cells (86 %), peaked in 4 - 6 min (Fig. 1d), was concentration-dependent (Fig. 1e) and reproducible. The IC50 for β-PEA and TYR were 43.8 ± 6.5 μM (n = 10) and 31.2 ± 3.3 μM (n = 12), respectively. The maximal inhibition caused by β-PEA (100 μM) was 41.5 ± 6.6 %, (n = 10) while that caused by TYR (100 μM) was 60.7 ± 4.5 % (n = 12, $p < 0.05$). $GABA_B$ IPSP amplitude was also irreversibly reduced by the adrenergic antagonist phentolamine (30 - 100 μM, Fig. 1c). Phentolamine has been recently shown to be a TAs agonist (Bunzow et al., 2001). The inhibition caused by phentolamine (100 μM) was 30 ± 6.7 %, (n = 4, $p < 0.05$). Interestingly, neither β-PEA nor TYR (100 μM) affected the amplitude of the evoked fast EPSP (Fig. 2a) or the $GABA_A$ mediated IPSP (Fig. 2b). In fact, the amplitude of the fast EPSP in control conditions (13.6 ± 2.3 mV) was unchanged in the presence of β-PEA (100 μM, 13.5 ± 2.7 mV, n = 5, p = 0.29) or TYR (100 μM, 12.2 ± 3 mV and, n = 5, p = 0.34), and the amplitude of the $GABA_A$ IPSP (13.1 ± 1.2 mV) was not significantly modified by TYR (100 μM, 11.7 ± 1.4 mV, n = 5, p = 0.19) or β-PEA (100 μM, 12.3 ± 1.1 mV, n = 5, p = 0.13).

Responses to β-PEA and TYR after catecholamine depletion. Because TAs have been proposed to act as false transmitters displacing catecholamines from intracellular stores (Janssen et al., 1999; Mundorf et al., 1999), we tested whether a depletion of DA stores by pretreating the rats with reserpine (5 - 8 mg/Kg i.p.) and perfusing the slices with carbidopa (300 μM) affects the reduction of the $GABA_B$ IPSP caused by β-PEA and TYR. Even under these conditions, that abolish the indirect

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DA-mediated effects these TAs on the dopaminergic cells (Geracitano et al., 2004), β-PEA and TYR still induced a clear-cut, reversible depression of the $GABA_B$ IPSP (Fig. 2c). In these conditions, the maximal inhibition caused by TYR (100 μM) was 32.1 ± 4.5 % (n = 7) while that caused by β-PEA (100 μM) was 41.5 ± 6.6 % (n = 7).

β-PEA and TYR modulate $GABA_B$ postsynaptic responses. We analyzed the effect of β-PEA and TYR on the $GABA_B$-mediated activation of the GIRK channel by using whole-cell patch-clamp recordings. Pressure applications of the $GABA_B$ agonist baclofen (100 μM, 1s, every 2 min) in the vicinity of the soma of the recorded neuron (voltage-clamped at - 60 mV) caused reproducible outward currents (135.3 ± 13.3 pA; n = 23, Fig. 3a). Bath application of β-PEA (100 μM) and TYR (100 μM) reversibly reduced the baclofen activated outward current (IBac) by 55.6 ± 3.7 % ($p < 0.001$; n = 18) and by 31.1 ± 3.9 % ($p < 0.01$; n = 10), respectively in 21 out of 23 cells tested (91 %). β-PEA and TYR did not affect IBac responses in 3 cells. The time-course of the effects of β-PEA and TYR on the IBac is shown in figure 3b. The maximal reduction of the amplitude of the IBac peaked after 4 - 6 min superfusion of β-PEA and 6 - 8 min superfusion of TYR. In addition, β-PEA and TYR caused a small inward current (35 ± 22 pA, n = 18) and did not modify membrane resistance. Bath application of DA (100 μM) (in the presence of DA antagonists) did not affect the baclofen response (n = 3, p = 0.77, data not shown). β-PEA and TYR also inhibited the outward current during continuous perfusion of baclofen (10 μM), in order to obtain a sustained activation of the GIRK channels. Thus, brief (3 min) co-application of β-PEA (100 μM) or TYR (100 μM) together with baclofen (10 μM), induced a reversible reduction of the outward current of 30.5 ± 8 % and 25 ± 6 % respectively (n = 3, Fig. 3c). Interestingly, bath applications of the TAs agonist, phentolamine (100 μM) reduced IBac by 62.5 ± 3.4 % within 8-10 minutes of perfusion. This effect did not reverse following 30 minutes of washout of the drug from the bath (n = 7, $p < 0.05$ Fig. 3a - b).

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β-PEA and TYR depress the interaction of $GABA_B$ Gi/0 with GIRK channels. To further analyze the mechanisms involved in the attenuation of GIRK by β-PEA and TYR, we loaded the dopaminergic neurons with the non-hydrolyzable GTP analog GTP-γ-S trilithium salt (GTP-γ-S, 0.3 mM), included in the intracellular solution of the patch pipette. The stimulation of the $GABA_B$ receptor leads to the substitution of GDP with GTP at the Gαi protein subunits thus, allowing the Gβγ subunits to dissociate and stimulate GIRK channels directly. The presence of GTP-γ-S means that the Gαi subunits cannot re-associate with the Gβγ subunits to reverse their activation of GIRK (Kandel, 2000). Whole cell perfusion with GTP-γ-S activated a mean outward current of + 205 ± 25 pA, (n = 6), that gradually occluded non-reversing responses to transient $GABA_B$ receptor activation, indicating the saturated activation of GIRK by Gβγ subunits (Fig. 4a - b). In these conditions the mean holding current (Vhold = - 60 mV) was + 221 ± 38 pA, (n = 13). A subsequent 4 - 6 minutes bath application of β-PEA (100 μM) or TYR (100 μM) on cells either loaded with GTP-γ-S or also treated with baclofen caused a sustained and non-reversible mean inward current of 202.5 ± 28 pA, (n = 7) and 228.2 ± 31 pA, (n = 12) respectively. These data indicate that β-PEA and TYR inhibited the GIRK channel which had been tonically activated by the presence of GTP-γ-S and/or $GABA_B$ receptor stimulation. Interestingly, the inward current induced by β-PEA and TYR did not reverse within the time-course of recordings (up to 20 minutes after washout), in contrast to the reversible effects of β-PEA and TYR using normal GTP (see Figs. 1-3). Following the β-PEA- and TYR-induced inward current in GTP-γ-S treated cells, further application of baclofen was without effect (see Fig. 4a-b, n = 11). This suggests that β-PEA and TYR inhibit GIRK through a G protein-dependent mechanism which cannot reverse due to the non-hydrolyzable nature of GTP-γ-S. The inhibition of a tonically activated GIRK conductance by β-PEA and TYR was supported by analysis of the current-voltage relationship (I-V). In neurons loaded with GTP-γ-S, current-voltage relationships were measured

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before and during bath application of the β-PEA and TYR. The net I-V for β-PEA (100 μM, Erev= - 102 ± 38 mV, n = 4) and TYR (100 μM, Erev= - 104 ± 26 mV, n = 4) indicate that trace amines specifically inhibit a potassium conductance (calculated reversal potential was - 105 mV, Fig. 4c). Consistent with the involvement of the GIRK channel, the β-PEA and TYR conductances showed inward rectification at hyperpolarized potentials (see non-linearity of I-V plot).

PKA, PKC, PLC and intracellular calcium changes do not mediate the effects of β-PEA and TYR. Activation of the TA receptor has been associated with the enhancement of intracellular levels of c-AMP (Borowsky et al., 2001; Bunzow et al., 2001). To investigate the possible role of a cAMP-PKA phosphorylation processes (Bonci and Williams, 1996; Couve et al., 2002) in the inhibition of baclofen responses by β-PEA and TYR, we tested blockers of the adenyl cyclase–cAMP–PKA pathway. Bath application of the adenyl cyclase inhibitor, SQ-22,536 (3 μM, 15 - 20 min), or the PKA-PKC inhibitor, staurosporine (1 μM, 15 - 20 min) or the intracellular dialysis of neurons with the PKA inhibitor c-AMPS-Rp, triethylammonium salt (Rp-cAMPS, 500 μM, 20 min) had no effect on the inhibition of the baclofen responses caused by either TYR (100 μM) or β-PEA (100 μM).

IBac was inhibited by β-PEA and TYR respectively: 53.7 ± 4.1 %, p = 0.34 and 24.5 ± 5.9 %, p = 0.54, n = 4 in SQ-22,536; 43.3 ± 3 %, p = 0.98 and 33 ± 3.1 %, p = 0.79, n = 4 in staurosporine; 38.3 ± 3.8 %, p = 0.59 and 25.8 ± 5.2 %, p = 0.53, n = 4 with S-Rp-cAMP.

We also considered the possibility that β-PEA and TYR inhibit GIRK channels via stimulation of PLC mediated pathways. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PtIns(4,5)P2) to yield the second messengers inositol(3,4,5)trisphosphate (InsP3) and diacylglycerol (DAG). The PLC inhibitor 1-[6-[((17β-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122, 20 μM, 12-16 minutes) superfused on the dopaminergic cells did not affect the reduction of IBac by β-PEA and TYR. In this condition IBac was inhibited by β-PEA and TYR respectively: 40.7 ± 3 %, p =

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0.7 and 32.7 ± 8.7 %, p = 0.82, n = 3. The activity of U73122 was verified by its inhibition of 72 ± 10 % (n = 4, p < 0.01, data not shown) of an outward current caused by puff applications of the selective mGluR-I agonist (S)-3,5- dihydroxyphenylglycine (DHPG) (100 μM, 50 ms). To investigate whether a PLC-induced depletion of PtIns(4,5)P2, which is thought to be required for activation of GIRK channels (Petit-Jacques et al., 1999; Sui et al., 1998), is involved in the TAs-induced depression of the $GABA_B$-activated GIRK channel, we loaded the cells with PtIns(4,5)P2 via the patch pipette (Meyer et al., 2001). Inclusion of PtIns(4,5)P2 (nominally 500 μM) had no effect on the amplitude or time course of IBac and did not significantly affect the β-PEA- and TYR-induced inhibition of the $GABA_B$-activated GIRK (35.7 ± 10.6 %, p = 0.63 and 34.7 ± 8.7 %, p = 0.58, n = 3).

Therefore, in line with the lack of effect of the PLC inhibitor U73122, there was no evidence that a depletion of PtIns(4,5)P2 is causally related to the β-PEA- and TYR-induced depression of the $GABA_B$-activated GIRK channel. Finally, a separate set of microfluorimetric experiments indicated that β-PEA and TYR did not modify the intracellular concentration of calcium. The mean ratio value for cytoplasmatic calcium remained unchanged in β-PEA (100 μM, Ca2+ ratio = 105.5 ± 9.6 % of control n = 4) or in TYR (100 μM, Ca2+ ratio = 109.8 ± 13.6 % of control, n = 4, not shown). All together, these results suggest that neither the activation of PKA, PKC, PLC nor changes in cytoplasmatic calcium level represent the signaling step mediating the inhibitory effects of β-PEA and TYR on the $GABA_B$ receptor–activated responses.

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Discussion

The results presented here demonstrate that β-PEA and TYR reversibly depress the slow $GABA_B$ IPSP in midbrain dopaminergic cells, mainly by inhibiting the $GABA_B$-receptor activated GIRK channels. Moreover, because this depression became irreversible in GTP-γ-S-treated cells, we identify a G-protein mediated role for β-PEA and TYR in modulating synaptic transmission in the mammalian central nervous system. Although, the mobilization of DA from intracellular stores by β-PEA and TYR could be an important factor for the inhibition of the $GABA_B$ IPSPs (Federici et al., 2002), DA did not mediate the action of TAs, because the β-PEA- and TYR-activated reduction in GIRK conductance was not mimicked by the superfusion of this catecholamine in the presence of classical DA antagonists. In fact, we demonstrate here that β-PEA and TYR still depressed the IPSP in reserpine- and carbidopa-treated cells in which the indirect (DA-mediated) effects of these TAs are abolished (Geracitano et al., 2004). Therefore β-PEA and TYR might activate specific TA receptors whose mRNA has been detected in the dopaminergic neurons of the ventral mesencephalon (Borowsky et al., 2001). The involvement of TA receptors is also supported by the observation that the unspecific and irreversible adrenergic antagonist phentolamine, mimicked the effects of β-PEA and TYR on the dopaminergic cells, consistent with its reported agonistic action on TA receptors (Bunzow et al., 2001). Phentolamine is not expected to evoke DA-mediated effects like β-PEA and TYR or α1-mediated effects (prazosin was present) and is thus likely to be acting through TA receptor activation. In control conditions, TYR was more potent than β-PEA in reducing the $GABA_B$ IPSP, while a reverse pattern of potency was observed for the TAs inhibition of GIRK currents. Interestingly, the effects of TYR and β-PEA on $GABA_B$ IPSPs following reserpine and carbidopa treatment showed a percent inhibition almost identical to the inhibition of baclofen activated postsynaptic currents. Thus, the

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difference in the TYR- and β-PEA-induced inhibition between synaptic and baclofen evoked currents, could be due to additional effects of released DA (Federici et al., 2002).

It is generally assumed that GABA acting on $GABA_B$ receptors dissociates the Gαi/0-βγ dimer causing GIRK channel activation (Huang et al., 1997; Kunkel and Peralta, 1995; Lewohl et al., 1999). Therefore, in cells loaded with the non-hydrolyzable GTP analogue, GTP-γ-S, the $GABA_B$ agonist, baclofen induced a sustained opening of the GIRK channels that was irreversibly suppressed by TYR and β-PEA. This supports the notion that a) the β-PEA and TYR inhibition of $GABA_B$ receptor-activated GIRK is a G protein-dependent process and b) the persistent closure of the GIRK channels by TYR and β-PEA bypasses events taking place upstream of the dissociation of the βγ subunits activated by $GABA_B$ receptors. Thus, the non-reversible effects of TYR and β-PEA in the presence of GTP-γ-S are not consistent with an action of these TAs directly at the $GABA_B$ receptor or a direct blockade of the GIRK channel which are not likely to be affected by GTP-γ-S.

Interestingly, the intracellular perfusion of GTP-γ-S initiated a slowly developing outward current in dopaminergic neurons even in the absence of exogenous agonist application. This implies that a tonic activated GIRK-linked receptors, due to spontaneous endogenous neurotransmitter release within the slice (e.g. $GABA_B$, nociceptin/orphanin FQ) (Lacey et al., 1988; Uchida et al., 2000), could be modulated by TYR and β-PEA.

The data presented here also demonstrate that PKA or PKC or PLC or an increase in $[Ca^{2+}]_i$ are not causally involved in the inhibitory actions of TYR and β-PEA on GIRK channels. We therefore reasoned that a negative modulation of the activation the K^+ channels could account for the TYR- and β-PEA-induced inhibition of the baclofen-induced current. A similar negative regulatory mechanism has recently been suggested for the orexin-induced depression of neurotransmitter-activated GIRK channels in mammalian neurons (Hoang et al., 2003).

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Therefore, the reduction of the $GABA_B$ IPSP might be principally due to the activation of G protein-coupled trace amine receptors that are stimulated by the trace amines TYR and β-PEA. Although the IC_{50} for the reducing effects of trace amines is above the nanomolar range usually found in the brain (Berry, 2004), it could be possible that, under particular metabolic conditions or pharmacological treatments (e.g. MAO inhibition), the synaptic activity of $GABA_B$ receptors is regulated by TAs. Accordingly, saturable, high affinity binding sites for *p*-[3H]tyramine (Ungar et al., 1977; Vaccari, 1986; Vaccari and Gessa, 1989) and β-[3H]PEA (Nguyen and Juorio, 1989) have been reported in the nigro-striatal system.

Functional implications. There are evidences suggesting that trace amines coexist with DA in the dopaminergic cells (Juorio et al., 1991) and activate motor activity by interacting with the dopaminergic system (Durden and Philips, 1980). The modulation of the GIRK channels by TAs might have a profound impact on integrative functions of the dopaminergic neurons to reduce the postsynaptic hyperpolarization caused by GABA released at inhibitory synapses. This suggests the involvement of trace amines in processes regulating motion, emotion and reward (Borison et al., 1977; Sabelli et al., 1978; White, 1996; Wise and Bozarth, 1987). In particular, the shaping of GABA inputs could be an important phenomenon for the development of addiction, since it has been recently demonstrated that drugs of abuse alter $GABA_B$ receptor transmission in the ventral midbrain (Giorgetti et al., 2002) and activate TAs receptors (Borowsky et al., 2001).

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Figure Legends

Figure. 1 TAs inhibit the $GABA_B$ IPSP. (a) β-PEA and (b) TYR reduce, in a concentration-dependent and reversible manner, the IPSP recorded with intracellular microelectrodes from DA neurons. (c) Phentolamine irreversibly reduces the IPSP (Each trace is an average of 4 sweeps). (d) Time course of the inhibition of $GABA_B$ IPSPs by β-PEA (100 μM , filled diamonds) and TYR (100 μM, white squares) applied for the time indicated by the bar. Each data point represents the averaged IPSPs taken from 8 - 12 cells, each of which was normalized to the average amplitude of the IPSPs preceding TA superfusion. (e) Concentration-response curves of inhibition of the $GABA_B$ IPSP are shown for β-PEA and TYR. Each point represents the mean ± S.E.M. of n = 12 cells. The membrane potential during these and the following intracellular experiments was maintained at - 65 ± 2 mV by injecting hyperpolarizing current. The apparent input resistance was 107 ± 9 MΩ (n = 12).

Figure. 2 TAs do not affect the ionotropic excitatory and inhibitory postsynaptic potentials but reduce the $GABA_B$ IPSP in DA depleted slices. Neither the fast EPSPs (a) nor the $GABA_A$ IPSPs (b) are modified by β-PEA and TYR. $GABA_A$ IPSPs in (b) are depolarizing due to the hyperpolarized membrane potential. (c) A DA-depleting treatment (reserpine 5 - 8 mg/Kg i. p. and superfused carbidopa, 300 μM for more than 30 min) does not affect the TAs-induced inhibition of the $GABA_B$ IPSP.

Figure. 3 TAs reduce the postsynaptic responses to $GABA_B$ receptor stimulation. (a) Outward currents caused by local pressure ejection applications of baclofen (puff Bac, arrows) are reversibly inhibited by β-PEA (100 μM, upper traces), TYR (100 μM, middle traces) and not reversibly inhibited by phentolamine (Phentol., 100 μM, lower trace). (b) Time course of the inhibition of the baclofen-

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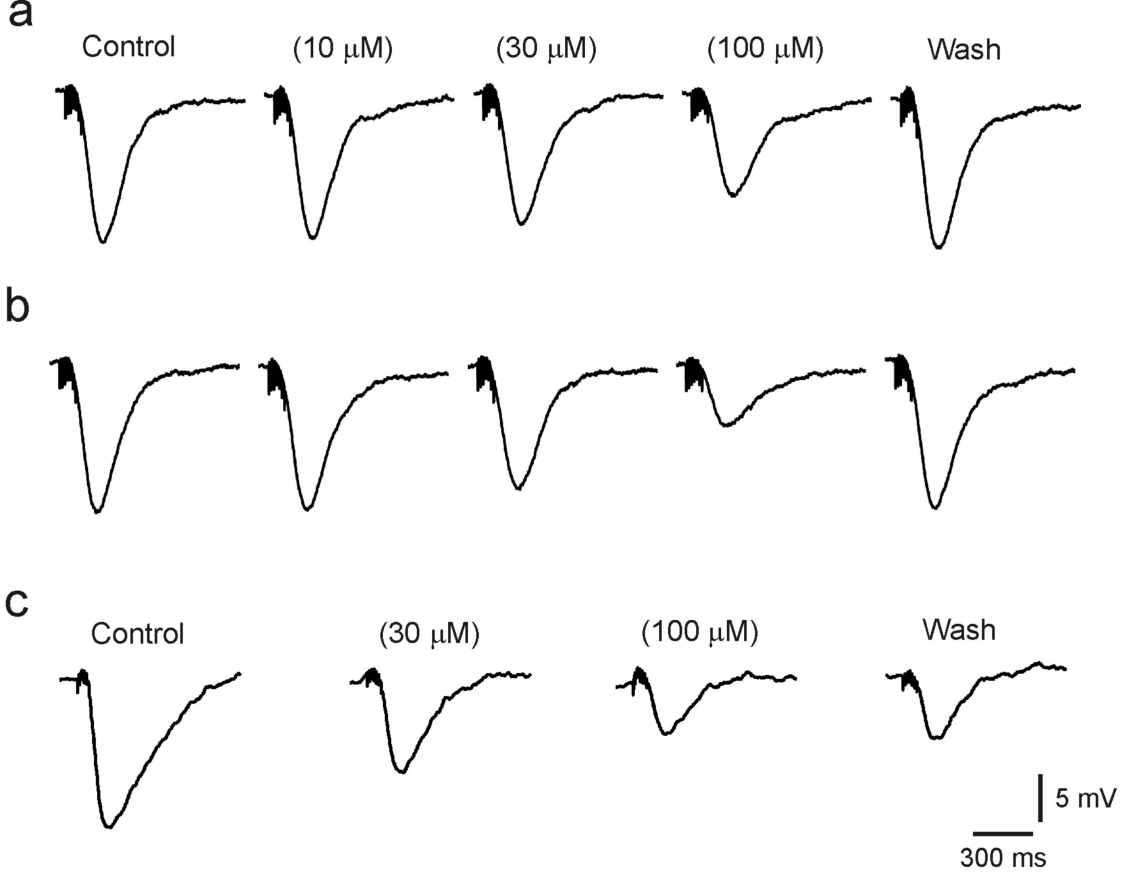
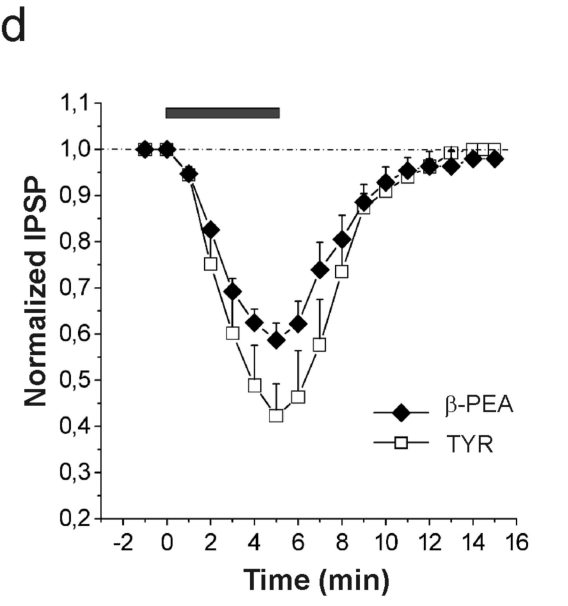
elicited outward current by β-PEA (100 μM, filled diamonds), TYR (100 μM, open squares) and phentolamine (100 μM, filled circles). Each point is obtained from 20 cells. (c) Reversible inhibition caused by a brief perfusion of both TAs on the outward current induced by a sustained bath application of baclofen on a dopaminergic neuron. The membrane potential during these and the following patch-clamp experiments was maintained at - 64 ± 2 mV.

Figure. 4 A G-protein-linked mechanism mediates the inhibitory effects of TAs on the activated GIRK. The traces in (a) show currents recorded immediately following the establishment of whole cell recordings. Intracellular solutions containing GTP-γ-S (0.3 mM) activate a sustained outward current that occludes the outward responses to pressure ejection of baclofen (Puff Bac arrows). Subsequent application of β-PEA (upper trace) or TYR (lower trace) inhibits the sustained outward current in an irreversible manner. (b) The histograms summarize the inhibition by TAs of the outward current elicited by GTP-γ-S and baclofen. Outward currents were measured relative to the holding current at the onset of whole cell recordings and normalized with respect to the outward current present prior to TA application. Numbers next to bars indicate the number of experiments. (c) Net current-voltage relationships (I-Vs) for β-PEA and TYR represent the difference between I-Vs measured at the peak of the outward current induced by GTP-γ-S and following the application of the TA. These I-Vs show a reversal potential consistent with E_K and inward rectification consistent with a reduction of GIRK.





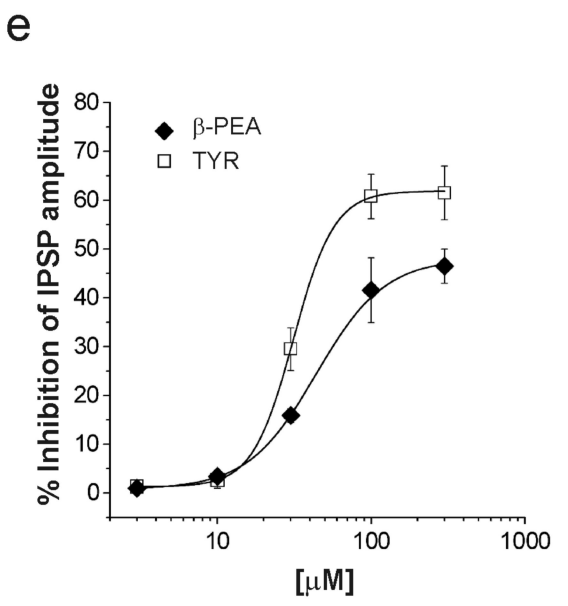


Fig. 1

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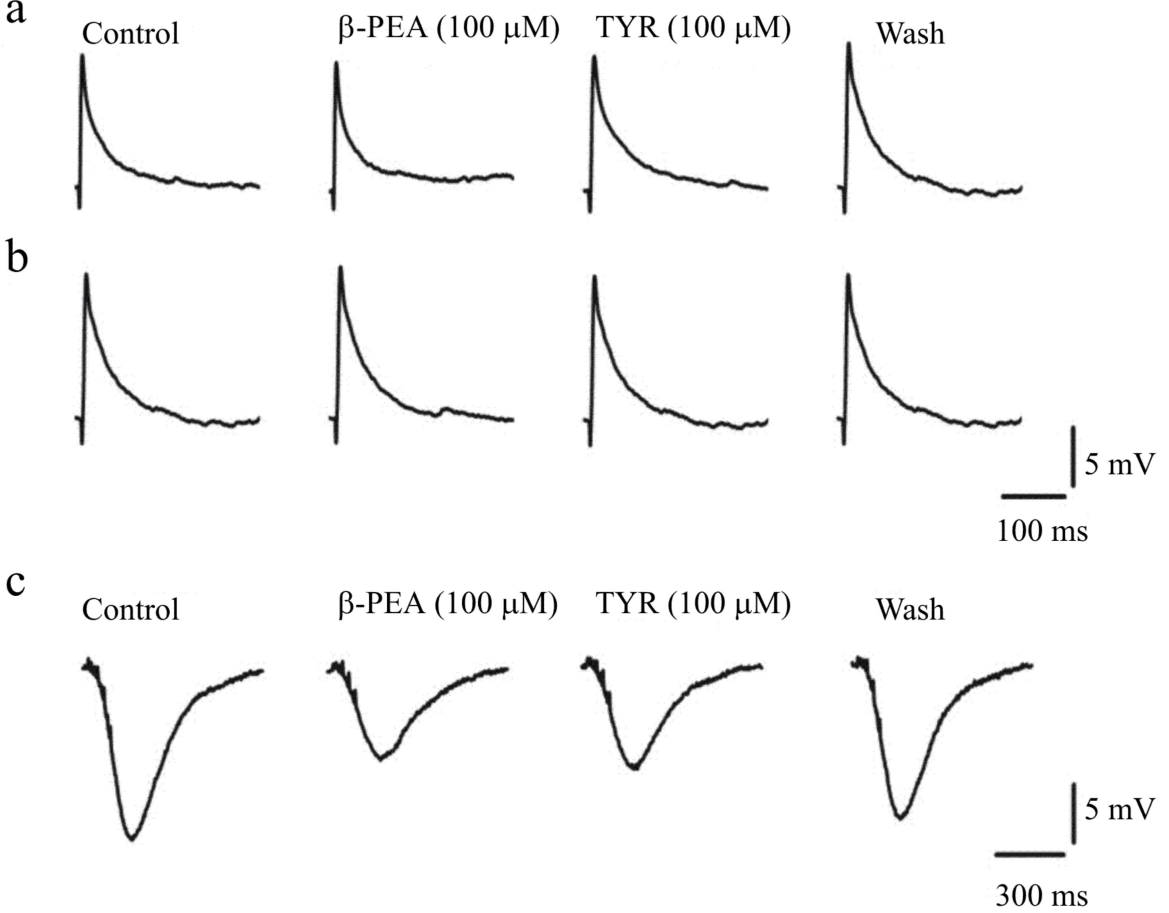
a
Control
β-PEA (100 μM)
TYR (100 μM)
Wash
b
5 mV
100 ms
c
Control
β-PEA (100 μM)
TYR (100 μM)
Wash
5 mV
300 ms

Fig. 2

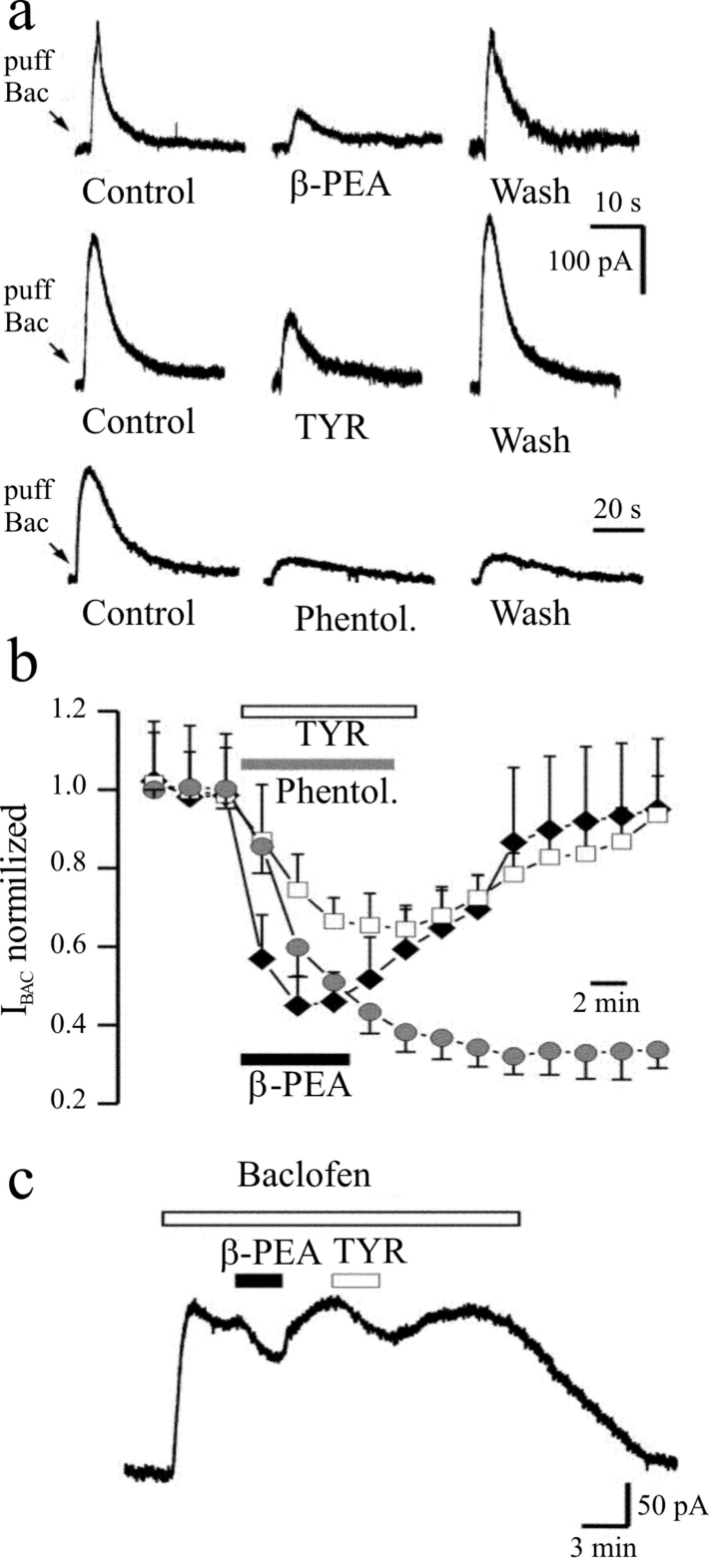
a
puff
Bac
Control
β-PEA
Wash
10 s
100 pA
puff
Bac
Control
TYR
Wash
puff
Bac
20 s
Control
Phentol.
Wash
b
1.2
1.0
0.8
0.6
0.4
0.2
I_BAC normilized
TYR
Phentol.
2 min
β-PEA
c
Baclofen
β-PEA TYR
50 pA
3 min

Fig. 3

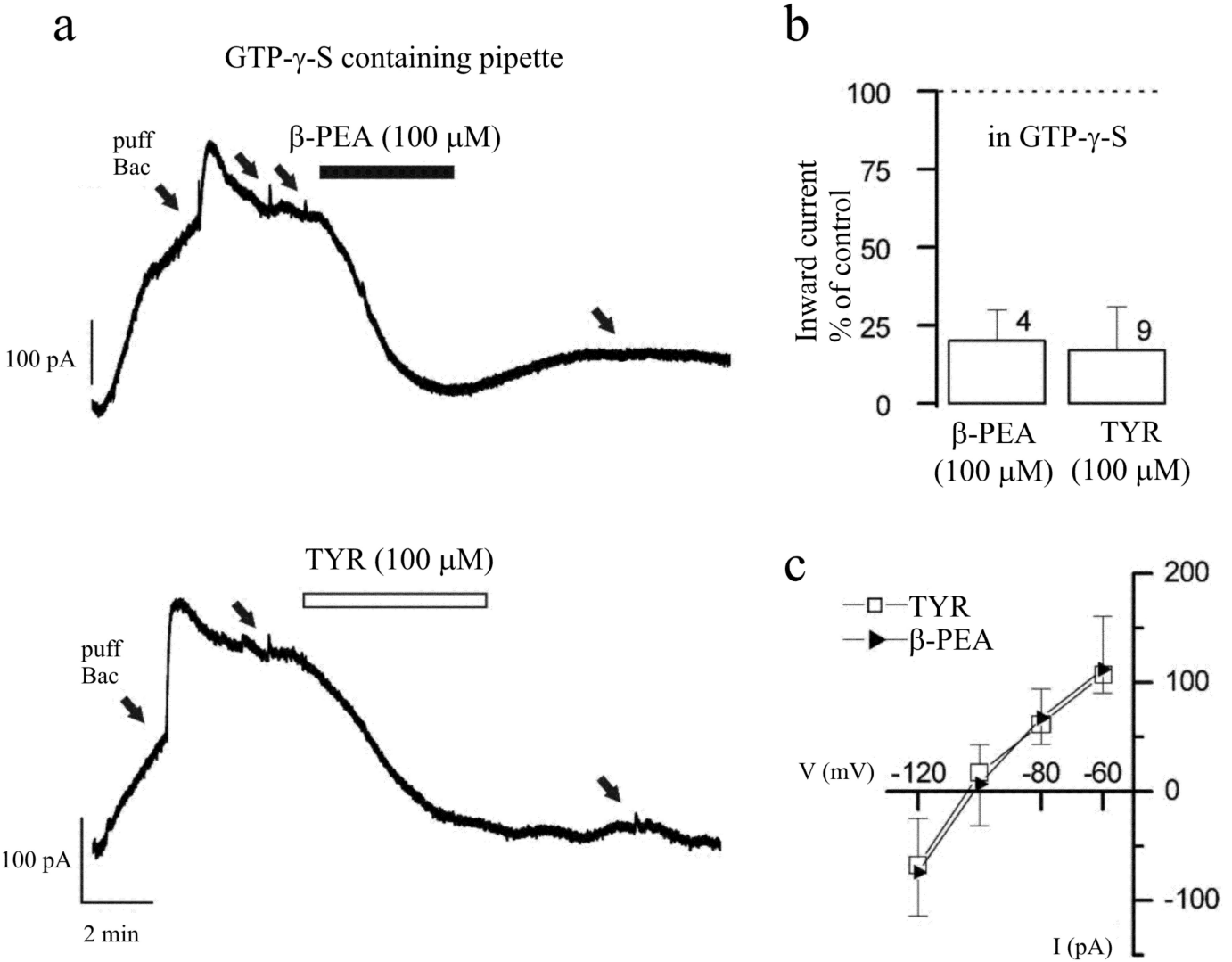
a
GTP-γ-S containing pipette
puff
Bac
β-PEA (100 μM)
100 pA
TYR (100 μM)
puff
Bac
100 pA
2 min
b
100
75
50
25
0
in GTP-γ-S
Inward current
% of control
4
9
β-PEA
(100 μM)
TYR
(100 μM)
c
TYR
β-PEA
200
100
0
-100
V (mV)
-120
-80
-60
I (pA)

Fig. 4