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Inversion of Ca^{2+} current modulation during recovery of neuroblastoma cells from pertussis toxin pretreatment

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Human neuroblastoma cells (SH-SY5Y) differentiated by retinoic acid exhibited high threshold-activated N-type Ca^{2+} currents, which were largely inhibited by the two enkephalins, DAGO and DPDPE, as well as by dopamine and somatostatin. The inhibitory effects were fully abolished after pretreatment of cells with pertussis toxin. After washing out the toxin, the inhibitory effects re-established with a time constant of about 16 h. The recovery of Ca^{2+} current inhibition was similar for all tested agonists. Unexpectedly, we observed a neurotransmitter-induced stimulation of Ca^{2+} currents in approximately 10% of all investigated cells during the recovery phase. Such a stimulatory effect by otherwise inhibitory receptors was never seen in control cells. It did also not occur when exogenous purified G-proteins of the G_i family were reconstituted via the patch pipette, suggesting that additional mechanisms may play a role in the appearance of stimulatory effects during the recovery phase after pertussis toxin pretreatment.

Ca^{2+} channel current; SH-SY5Y; Neuroblastoma cell; Opioid; Pertussis toxin; G-protein

1. INTRODUCTION

Voltage-dependent Ca^{2+} channels play a prominent role in the regulation of transmitter secretion [1], while Ca^{2+} channels themselves are under tight control of receptors which recognize transmitters and are functionally coupled to channels via G-proteins [2]. One of the best studied examples for receptor-dependent modulation of Ca^{2+} channels is the clonal pituitary cell line, GH_3. Using antisense oligonucleotides as probes to specifically suppress the expression of G-proteins, we recently demonstrated that the inhibitory receptors for somatostatin (SST) and acetylcholine (via M_4 receptors) distinguish between the two splicing variants of α_o, as well as the β and γ subunits [3,4]. Thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone and angiotensin II stimulated Ca^{2+} channels via G_{i2} when simultaneous protein kinase C activation occurred [5,6]. All modulatory effects were abolished by pretreatment of cells with pertussis toxin (PTX). This exotoxin of *Bordetella pertussis* ADP-ribosylates the α subunits of G-proteins of the G_i and G_o families, as well as the transducins making them unaccessible for activated receptors [7]. In the present study we investigated the recovery of inhibitory modulations after washing out PTX. We performed the experiments on human neuroblastoma SH-SY5Y cells, which are closely related to human peripheral sympathetic ganglion cells [8]. SH-SY5Y cells exhibit receptors, including μ and δ opioid receptors [9,10], as well as receptors for somatostatin (SST, receptor subtype not identified) and dopamine (DA, presumably receptors of subtype D_2) which strongly inhibit N-type Ca^{2+} channels in a PTX-sensitive manner [11,12]. Here we report that after pretreatment with PTX at least 90 h were necessary for a full recovery of the inhibitory effects on the Ca^{2+} current. Since the ADP-ribosylation of G-protein α subunits is quasi-irreversible, the reappearance of receptor-dependent inhibition is supposed to reflect the rate of G-protein resynthesis. Unexpectedly, in about 10% of cells investigated during the recovery phase we observed stimulatory effects by otherwise inhibitory agonists.

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Abbreviations. DAGO, [D-Ala2, *N*-Me-Phe4, Gly5-ol]-enkephalin; DPDPE, [D-Pen2, Pen5]-enkephalin; TRH, thyrotropin-releasing hormone; DA, dopamine; SST, somatostatin; PTX, pertussis toxin, an exotoxin of *Bordetella pertussis*; PI response, phosphatidylinositol 4,5-biphosphate hydrolysis.

2. MATERIALS AND METHODS

2.1. *Culturing of cells*

SH-SY5Y cells (clonal human neuroblastoma cells) were grown in non-confluent monolayer cultures. Neuronal differentiation was induced by exposure of cells to retinoic acid (10 μM) for 5 days in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humified atmosphere with 7% CO_2 at 37°C. The differentiation resulted in a reduction of cell division, neurite extension and increase in Ca^{2+} current [11]. SH-SY5Y cells were pretreated for 24 h with 200 ng/ml PTX. For determination of the recovery from PTX action, cells were washed twice in PTX-free medium and kept in PTX-free RPMI-1640 medium (plus supplements) for various times before use for electrophysiological measurements.

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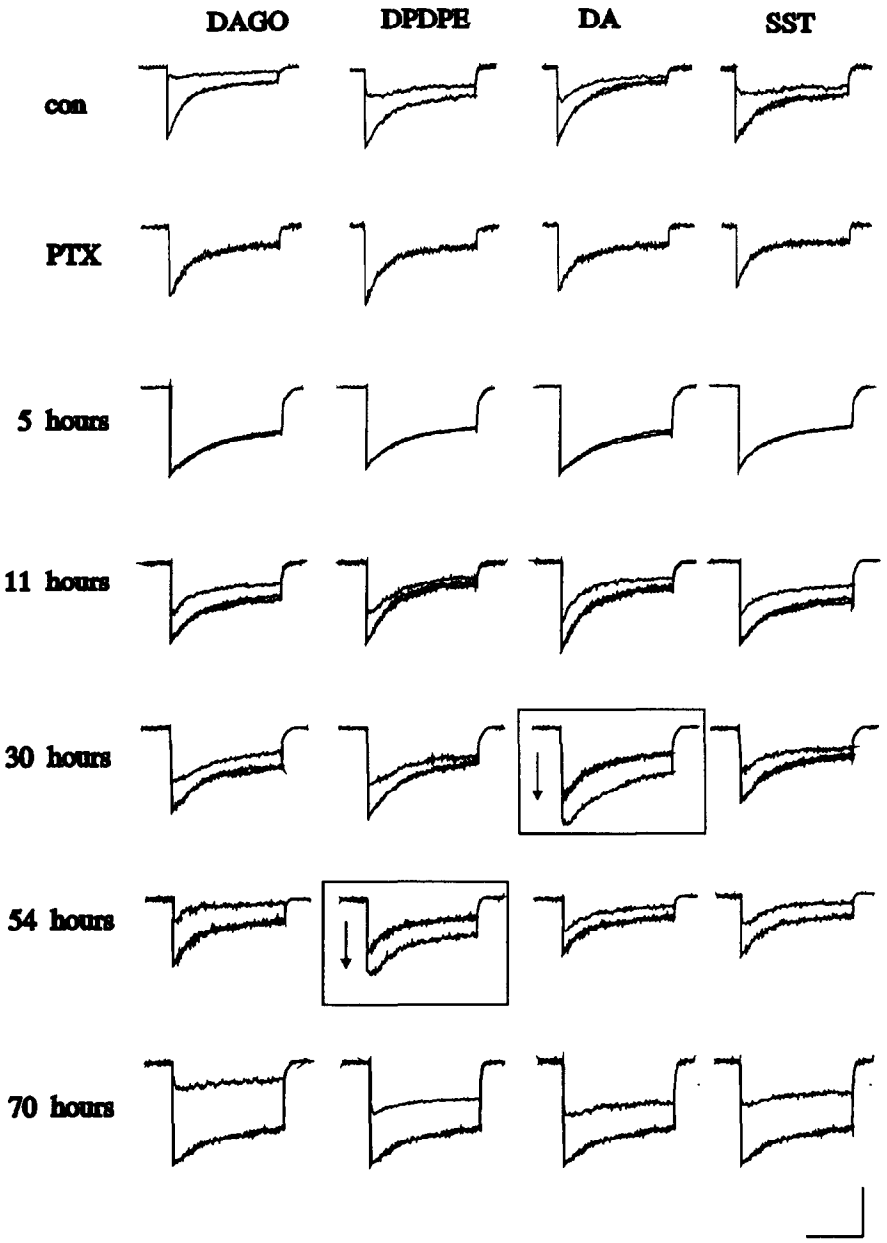

Fig. 1. Recovery of inhibitory effects after PTX pretreatment of SH-SY5Y cells. Shown are superimposed traces of Ca^{2+} currents recorded during voltage-clamp pulses from −80 mV to 0 mV under control conditions, after application of DAGO (1 μM), DPDPE (1 μM), DA (30 μM) and SST (1 μM) and after washing-out the agonists. Traces in the first line refer to a control cell, traces in the second line to a cell after PTX pretreatment (200 ng/ml for 24 h). Panels 3–7 demonstrate current traces recorded from cells at defined times after washing-out PTX (times are given on the left; medium, RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin/streptomycin). Stimulatory events are marked by frames and arrows. All traces within one horizontal panel were recorded from one cell. The vertical and horizontal calibration bars represent 50 pA and 50 ms, respectively.

2.2. *Purification of G-proteins*

G-protein α_i-subtypes were purified chromatographically from brains (α_{i1}, α_{i2}) or human thrombocytes (α_{i2}, α_{i3}) as described elsewhere [13]. In brief, cholate extracts from cell membranes were subsequently passed over ion-exchange, gel-filtration and hydrophobic interaction columns. Mixtures of isolated α-subunits were then separated by fast protein liquid chromatography (FPLC) over Mono Q columns and identified by subtype-specific antibodies. The purity of individual G-protein α_i-subunits was greater than 95% as determined by silver staining of polyacrylamide gels and GTPγS binding. Mixtures of α_{i1}, α_{i2} and α_{i3} were immediately diluted prior to use with pipette solution supplemented with Lubrol PX (0.05%) and BSA (0.5%) to a final concentration of 40 nM each and infused into SH-SY5Y cells.

2.3. *Patch-clamp recordings*

Voltage-sensitive inward currents were recorded using the whole-cell patch-clamp technique [14]. The recording patch pipette contained (in mM): 110 $CsCl_2$, 1 $MgCl_2$, 3 MgATP, 10 EGTA, 10 CsHEPES (pH 7.4, adjusted with CsOH). During electrophysiological measurements, cells were superfused at 37°C with a solution containing (in mM): 125 NaCl, 10.8 $BaCl_2$, 1 $MgCl_2$, 5.4 $CsCl_2$, 10 glucose, 10 NaHEPES (pH 7.4, adjusted with NaOH). Ba^{2+} was used as the charge carrier in order

to increase currents through Ca^{2+} channels and to block K^+ currents. Ba^{2+} currents through Ca^{2+} channels were recorded under voltage-clamp conditions during 100 ms long pulses from −80 mV to 0 mV. Altogether 121 cells were investigated.

3. RESULTS AND DISCUSSION

SH-SY5Y cells differentiated for 5 days with retinoic acid exhibited Ba^{2+} currents through voltage-dependent Ca^{2+} channels of 25.3 ± 7.03 pA/pF amplitude when voltage-clamped from −80 mV to 0 mV. The inward currents were fully blocked by ω-conotoxin (1 μM) but only slightly affected by the L-type Ca^{2+} channel blocker and opener, PN200-110 (1 μM) and BayK8644 (1 μM), respectively, suggesting that SH-SY5Y cells preferentially express high-threshold N-type Ca^{2+} channels. Superfusion of cells with the μ-opioid agonist, DAGO (1 μM), the δ-opioid agonist, DPDPE (1 μM), as well as with DA (30 μM) and SST (1 μM), caused a strong inhibition of the Ca^{2+} current (Fig. 1). The mean inhibition (± S.E.M.) amounted to 77.6 ± 2.5% (n = 10), 63.9 ± 2.0% (n = 12), 59.9 ± 2.0% (n = 9) and 63.7 ± 3.8% (n = 10) for DAGO, DPDPE, DA and SST, respectively. All inhibitory effects occurred within seconds and were fully reversible on washing-out the agonists. The effects of DAGO and DPDPE were antagonized by naloxone (10 μM). In addition to the inhibition, the neurotransmitters induced an apparent slowing of the time-course of activation, presumably due to

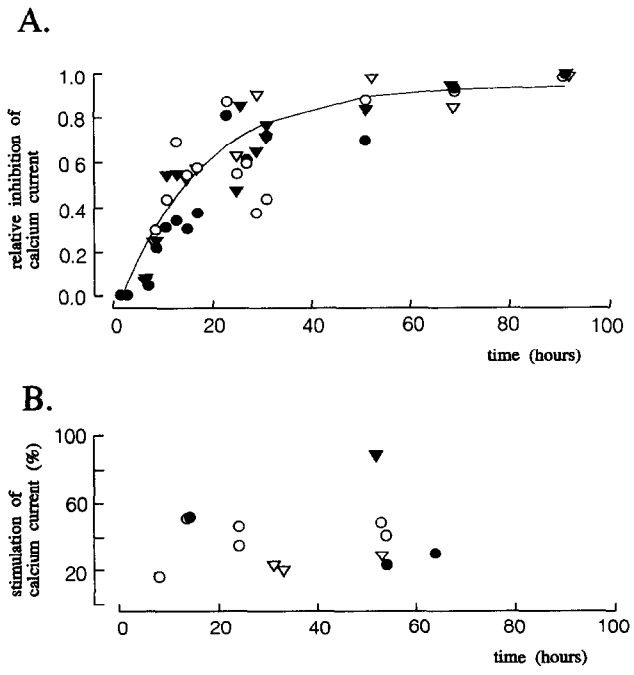

Fig. 2. Time-course of recovery of Ca^{2+} current modulation after PTX pretreatment. (A) The recovery of inhibition is given with reference to the mean inhibition measured in control SH-SY5Y cells. The different symbols represent different agonists: DAGO (○), DPDPE (●), DA (▽), SST (▼). The data were fitted according to $f(t) = f_{max} - \exp(-t/\tau)$, with τ = 16.6 h and f_{max} = 0.9. (B) Stimulatory events are shown as percentage increase of the control current. The same symbols as in A are used.

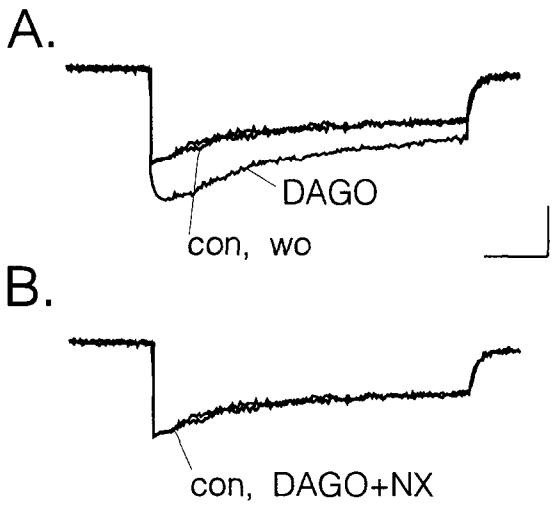

Fig. 3. Blockade of opioid-induced stimulation of Ca^{2+} current by naloxone. The current traces were recorded during voltage-clamp pulses from −80 mV to 0 mV. Con refers to control traces, DAGO to the traces recorded under superfusion with DAGO, DAGO + NX to traces after superfusion of DAGO plus naloxone and wo to traces after washing-out the agonist. While DAGO caused the stimulation in A, it had no effect if naloxone was superfused in addition (B, same cell as in A). The vertical and horizontal calibration bars represent 50 pA and 50 ms, respectively.

a voltage-dependent unblocking of G-proteins [15]. Pretreatment of SH-SY5Y cells with 200 ng/ml PTX for 24 h resulted in the abolishion of the effects of DAGO, DPDPE, DA and SST (Fig. 1).

To functionally determine the rate of synthesis of G-proteins, we used the fact that PTX irreversibly ADP-ribosylates G-proteins [16]. PTX-pretreated SH-SY5Y cells were re-incubated for variable times in PTX-free medium (plus supplements) before measuring the effects of DAGO, DPDPE, DA and SST. The re-establishment of modulation was assumed to represent the recovery of G-proteins in the signal transduction process. The measured inhibitory neurotransmitter effects were referred to the mean effects observed by the respective agonists in control experiments (Fig. 2). The phase of re-appearance of inhibitory effects could be roughly described by a monoexponential process with a time constant of approximately 16 h. After 90 h, the effects were fully restored for all the agonists tested. These times are in the range of values reported previously. By determining the degradation rate of radioactively labeled G-proteins, Silbert et al. [17] suggested a turnover rate for G_o of about 60 h in GH_4 and more than 140 h in rat cardiomyocytes. Similar times were also observed after injection of antisense oligonucleotides suppressing different G-protein subunits [3]. During the phase of recovery the slowing of activation was less pronounced compared to control cells. Within a given cell the inhibitory effects varied considerably from agonist to agonist, suggesting a different efficiency of G-protein resynthesis for the different receptors.

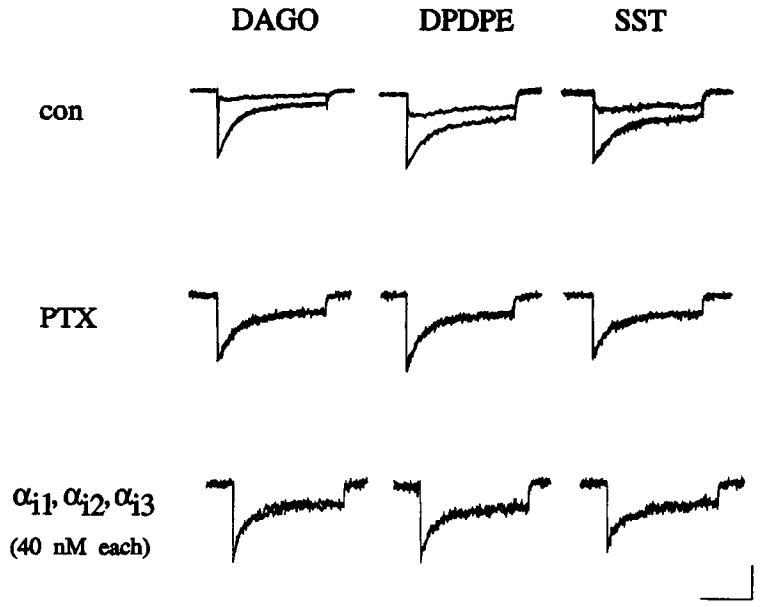

Fig. 4. Reconstitution of G_i into PTX-pretreated SH-SY5Y cells. Shown are superimposed traces of Ca^{2+} currents recorded under the same conditions as given in the legend to Fig. 1. Traces in the first line refer to a control cell, traces in the second line to a cell after PTX pretreatment and traces in the third line to a PTX-treated cell after infusion of a mixture of α_{i1}, α_{i2} and α_{i3} (40 nM each). The vertical and horizontal calibration bars represent 50 pA and 50 ms, respectively.

Unexpectedly, about 10% of all cells tested (n = 121) responded to the inhibitory agonists (same concentrations) with stimulations of the Ca^{2+} channel current. These stimulatory events occurred during the first 60 h of recovery (see Fig. 2B) and, for a given cell, was not seen simultaneously for all agonists (see Fig. 1, panels 5 and 6). No preference for a certain agonist could be detected. The stimulations ranged from 20% to 90% of controls (n = 13). Similar to the inhibitory effects, the stimulations occurred fast and were fully reversible on washing-out the agonists. The stimulatory effects were obviously mediated by receptors since they could be antagonized by the respective receptor antagonists. In the case of stimulations of Ca^{2+} channel currents by DAGO, naloxone (10 μM) completely abolished the effect in the same cell (Fig. 3). So far, a G-protein-dependent stimulation has only been described for endocrine cells where it is mediated by G_{i2} [5,6,18] and for cardiac and skeletal muscle where it is mediated by G_S [19]. Typically, these responses are induced by agonists inducing a PI response (e.g. TRH, angiotensin II) or stimulating adenylyl cyclase (e.g. β-adrenergic agonists) but never by inhibitory agonists such as opioids, DA and SST. It may be speculated that during the process of re-synthesis, 'wrong G-proteins' reconstitute which would then mediate the inverse modulatory effect. Since by using the [α-^{32}P]GTP azidoanilide-labelling method opioid agonists and SST have been demonstrated to activate both G_o and G_{i2} in a neuronal cell line [20], it is plausible that G_{i2} recovers faster than G_o, resulting in a preferential coupling of the inhibitory receptors to G_{i2}, leading to the observed stimulatory effects. To test this hypothesis, we directly reconstituted a mixture of purified α_{i1}, α_{i2} and α_{i3} via the patch-pipette into SH-SY5Y cells (Fig. 4). However, the exogenous G-proteins never resulted in stimulatory responses by DAGO, DPDPE or SST. Therefore additional mechanisms may play a role in the appearance of stimulatory effects during the recovery phase after PTX pretreatment. A possible pathway for activation of protein kinase C, which was found to be essential for the TRH-dependent stimulation of the Ca^{2+} current in GH_3 cells [6], may be due to serum factors.

The stimulatory effects seen during the re-synthesis phase after washing out PTX demonstrate a high variability of receptor–effector coupling on the level of G-proteins [21], which may play a physiological role in the modulation of synaptic activity during differentiation or slowly developing adaptation processes. In addition to the well-known modulatory events regulating voltage-operated Ca^{2+} channels [22,23], they may contribute to the plasticity of synaptic transmission.

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