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The heterotrimeric G protein Go_2 regulates catecholamine uptake by secretory vesicles

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Secretory vesicles store neurotransmitters that are released by exocytosis. Their membrane contains transporters responsible for transmitter loading that are driven by an electrochemical proton gradient across the vesicle membrane. We have now examined whether uptake of noradrenaline is regulated by heterotrimeric G proteins. In streptolysin O-permeabilized PC 12 cells, GTP-analogues and AlF_4^- inhibited noradrenaline uptake, an effect that was sensitive to treatment with pertussis toxin. Inhibition of uptake was prevented by Gαo-specific antibodies and mimicked by purified activated $G\alpha o_2$. No effect was seen when $G\alpha o_2$ in its inactive GDP-bound form or purified activated $G\alpha o_1$, $G\alpha i_1$ and $G\alpha i_2$ were tested. Down-regulation of uptake remained unchanged when exocytosis was inhibited by the light chain of tetanus toxin. Vesicular acidification was not affected whereas binding of [^{3}H]reserpine was reduced by GTPγS and $G\alpha o_2$. These data suggest that the monoamine transporter rather than the vacuolar ATPase is affected. We conclude that catecholamine uptake is controlled by $G\alpha o_2$, suggesting a novel function for heterotrimeric G proteins in the control of neurotransmitter storage.

Keywords: catecholamine uptake/heterotrimeric G protein Go/PC 12 cells/secretory vesicles/signal transduction

Introduction

Neurotransmitters are concentrated in the cytoplasm of neurones and neuroendocrine cells by biosynthesis or re-uptake before they are packaged into secretory vesicles. Two distinct transport activities are responsible for the uptake of neurotransmitters such as acetylcholine, amino acid transmitters and catecholamines. Specific transporters in the plasma membrane terminate the action of neurotransmitters by re-uptake into the presynaptic terminal (Schloss *et al.*, 1994) and vesicular transporters mediate the filling of secretory vesicles with neurotransmitter for a new round of secretion (Maycox *et al.*, 1990; Liu and Edwards, 1997).

Similar to the uptake of other non-peptide transmitters, the uptake of catecholamines into secretory vesicles is driven by an electrochemical proton gradient that is generated by a vacuolar proton ATPase residing in the vesicular membrane (Schuldiner *et al.*, 1978; Johnson, 1988). An electrogenic carrier, the monoamine transporter, exchanges two lumenal protons for one cytoplasmic amine molecule (Schuldiner *et al.*, 1978; Johnson, 1988). Two different monoamine transporters have been identified and termed VMAT1 and VMAT2, that differ in their tissue distribution and their substrate preference (Liu *et al.*, 1992; Stern-Bach *et al.*, 1992; Kreijci *et al.*, 1993; Liu and Edwards, 1997).

While the bioenergetic dependence of vesicular monoamine transport on the electrochemical proton gradient is well understood, it is presently not known whether uptake is regulated by other mechanisms. Regulation of vesicular loading in response to external stimuli or intracellular signals, however, could represent an important means for the control of transmitter output and thus may contribute to neuronal plasticity. Indeed, there is a large body of evidence suggesting that vesicular content is variable and may be subject to both short-term and long-term regulation (Van der Kloot, 1991). For these reasons, we have asked whether vesicular monoamine uptake is linked to intracellular signalling pathways that modulate its activity.

Our search for such regulatory mechanisms was guided by our recent observation demonstrating that heterotrimeric G proteins consisting of an α-, a β- and a γ-subunit are associated with small synaptic vesicles from brain and chromaffin granules from the adrenal medulla. Whereas on small synaptic vesicles both Go- and Gαi-subunits were detected, chromaffin granules contain only Gαo-subunits (Ahnert-Hilger *et al.*, 1994).

In the present study, we have investigated whether α-subunits of heterotrimeric G proteins regulate vesicular monoamine uptake. As a model system, we used rat phaeochromocytoma (PC 12) cells which store catecholamines in secretory vesicles and release them upon stimulation by Ca^{2+}-dependent exocytosis (Ahnert-Hilger *et al.*, 1989a,b). PC 12 cells can be selectively permeabilized using streptolysin-O (SLO) (Weller *et al.*, 1996) that generates pores in the plasma membrane large enough for the free passage of proteins and thus also allows direct access to secretory vesicles (Bhakdi and Tranum-Jensen, 1987; Ahnert-Hilger *et al.*, 1989a,b). Measuring vesicular uptake in permeabilized cells offers the unique advantage that lengthy vesicle purification procedures are avoided which may lead to the loss of regulatory factors, and vesicular transmitter uptake can be investigated under conditions close to those of intact cells.

We report here that vesicular catecholamine uptake is significantly down-regulated by factors activating hetero-

trimeric G proteins, an effect that was attributed to a selective activation of Gαo$_2$. Furthermore, we show that down-regulation is due to a direct control of the transporter rather than to a reduction of the electrochemical proton gradient.

Results

Vesicular monoamine uptake of PC 12 cells is down-regulated by activation of Gαo$_2$

PC 12 cells were suspended in a potassium-rich buffer and permeabilized with SLO according to standard procedures (see Materials and methods). When [^{3}H]noradrenaline was added to permeabilized cells, ATP-dependent uptake was observed (Figure 1A). As expected for the vesicular monoamine transport system, uptake was inhibited by reserpine (Figure 1A) and by agents like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) dissipating the electrochemical proton gradient (Johnston, 1988; Hell *et al.*, 1990; data not shown). To investigate whether heterotrimeric G proteins are involved in the regulation of catecholamine uptake, non-hydrolysable GTP-analogues (GTPγS or GMppNp) which activate G proteins were added, as was aluminium fluoride (AlF_4^-) which stabilizes a transition state of Gα corresponding to the GTP-bound form (Charbre, 1990). A significant inhibition of noradrenaline uptake was observed (25–50% of control; Figure 1A–C). GDPβS, which stabilizes the GDP-bound inactive conformation, had no effect. To clarify the nature of the responsible G protein, cells were treated with pertussis toxin before permeabilization. Pertussis toxin acts selectively on trimeric G proteins of the Gi/Go subclass, interrupts receptor G protein interactions, and interferes with effector modulations due to activated Gi/Go proteins (Jakobs *et al.*, 1984). As shown in Figure 1B, GTP-analogues failed to inhibit noradrenaline uptake when the cells were pretreated with pertussis toxin.

The experiments described so far suggest that vesicular catecholamine uptake is down-regulated by activation of a trimeric G protein belonging to the Gi/Go subfamily. To identify the responsible isoform and subunit, two independent and complementary approaches were used. First, affinity-purified polyclonal antibodies that recognize all known Gαo-subunits were added immediately after permeabilization. When the cells were subsequently incubated with AlF_4^-, noradrenaline uptake was not inhibited (Figure 1C). Second, we investigated whether the inhibition of noradrenaline uptake could be mimicked by addition of purified activated α-subunits of pertussis toxin-sensitive Gi/Go proteins. Permeabilized PC 12 cells are ideally suited for this experiment since the proteins can be introduced into the cytoplasm by adding them to the incubation mixture. α-Subunits of Gi$_1$, Gi$_2$, Go$_1$ and Go$_2$, purified from bovine brain (Figure 2A), were applied in their non-activated GDP-bound, activated GTPγS-bound and AlF_4^--activated forms, respectively. An inhibition was

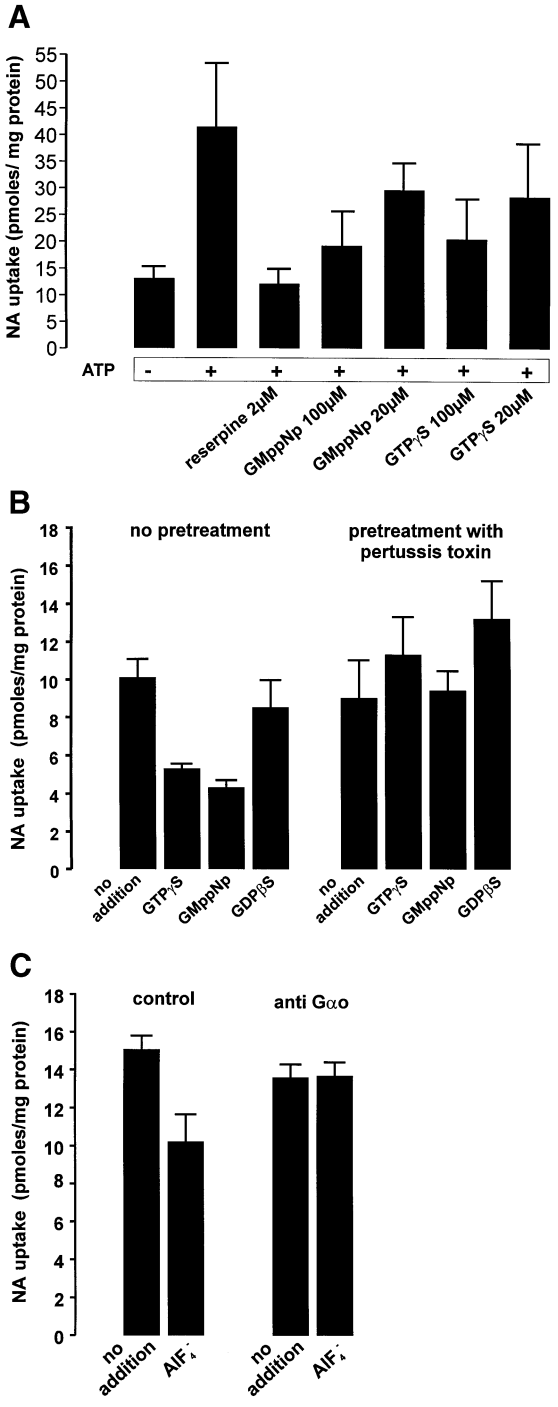

Fig. 1. Activators of heterotrimeric G proteins inhibit ATP-dependent, reserpine-sensitive uptake of [^{3}H]noradrenaline (NA) by permeabilized PC 12 cells. (**A**) SLO-treated and washed cells (see Materials and methods) were resuspended in KS-buffer containing either no MgATP, 2 mM MgATP or 2 mM MgATP with additions as indicated and incubated for 15 min at 36°C. The ATP-dependent noradrenaline uptake was completely blocked by the addition of 2 µM reserpine. Addition of the GTP-analogues GMppNp and GTPγS inhibited catecholamine uptake in a dose-dependent manner. (**B**) Inhibition of noradrenaline uptake by 50 µM GTPγS or 50 µM GMppNp was prevented by preincubating the cells with pertussis toxin. In contrast, 50 µM GDPβS did not affect catecholamine uptake. Unspecific accumulation (pmol/mg protein) in the absence of ATP was 4.2 ± 0.8 and 5.7 ± 0.6 for untreated or pertussis toxin-treated samples, respectively. (**C**) Following incubation with SLO, the cell pellets, were resuspended in 20 µl KS-buffer in the presence or absence of an affinity-purified antiserum (AS 6) specific for Gαo-subunits and incubated for 20 min. After removal of this solution fresh buffer containing MgATP and AlF_4^- (10 mM NaF and 50 µM $AlCl_3$). AlF_4^- inhibited catecholamine uptake. This inhibition was overcome by the antiserum specific for Gαo-subunits. Unspecific accumulation in the presence of 2 µM reserpine was 9.4 ± 1 pmol/mg protein. Values (n = 3, ± SD) are corrected for uptake in the absence of ATP (B) or in the presence of reserpine (C).

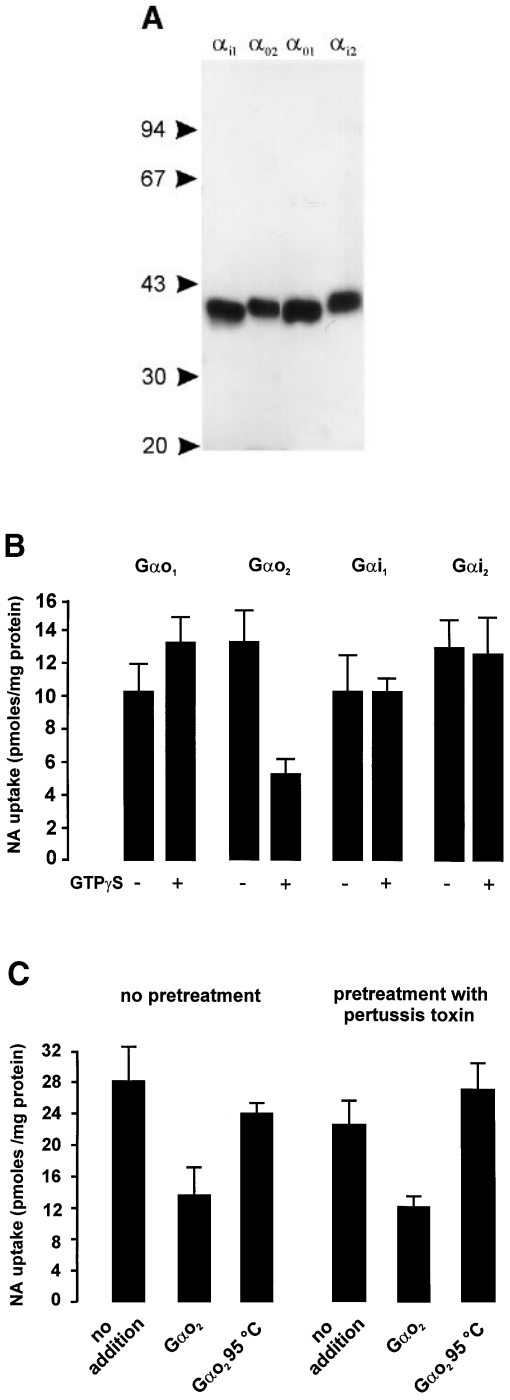

Fig. 2. Purified GTPγS-activated $G\alpha o_2$ but no other purified pertussis toxin-sensitive Gα-isoform inhibits ATP-dependent [^{3}H]noradrenaline uptake by permeabilized PC 12 cells. (**A**) SDS–PAGE and silver staining of purified Gα-subunits from bovine brain shows a single band of each protein. (**B**) The experimental design was the same as in Figure 1A. Gα-isoforms (20 nM final concentration) were added in the GTPγS-activated or in the inactive GDP-bound form. The isoforms were activated by the addition of 10 μM GTPγS and diluted 10- to 100-fold before the experiment. Only the activated $G\alpha o_2$-subunit inhibited catecholamine uptake. Unspecific accumulation in the absence of ATP was 5.3 ± 0.5 pmol/mg protein. (**C**) Pretreatment with pertussis toxin (100 ng/ml) did not prevent down-regulation caused by purified $G\alpha o_2$-subunit (10 nM final concentration). An aliquot of GTPγS-activated $G\alpha o_2$-subunit was denatured by heating for 30 min at 95°C before the experiment. Unspecific accumulation (pmol/mg protein) in the absence of ATP was 12.3 ± 1 and 14.1 ± 2 for untreated or pertussis toxin-treated samples, respectively. Values (n = 3, ± SD) represent ATP-dependent uptake.

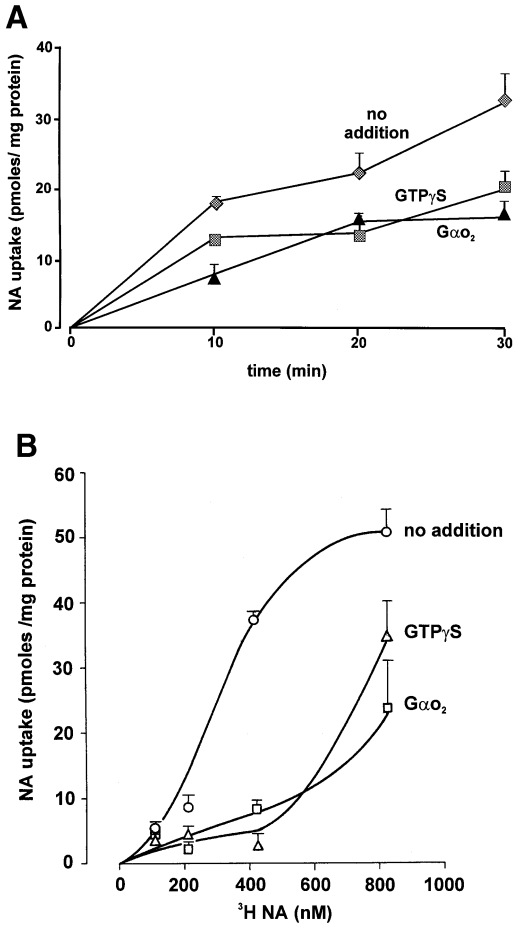

Fig. 3. GTPγS and $G\alpha o_2$ decrease catecholamine uptake by decreasing substrate affinity. (**A**) Time course of noradrenaline uptake. Permeabilized and washed PC 12 cells were incubated with KG-buffer plus 2 mM MgATP containing 50 μM GTPγS or 15 nM AlF_4^--activated $G\alpha o_2$. Unspecific accumulation (pmol/mg protein) in the presence of 2 μM reserpine was 6.9 ± 0.7, 9.6 ± 0.4 and 12.3 ± 0.5 for 10, 20 and 30 min, respectively. (**B**) Concentration–response of noradrenaline uptake. Permeabilized and washed PC 12 cells were incubated for 20 min with KG buffer plus MgATP with increasing concentrations of [^{3}H]noradrenaline. Substrate affinity decreased in the presence of 50 μM GTPγS or 15 nM AlF_4^--activated $G\alpha o_2$. Unspecific accumulation (pmol/mg protein) in the presence of 2 μM reserpine was 3.6 ± 0.4, 4.6 ± 2.1, 11.5 ± 1.8 and 32 ± 2.1 for 100, 200, 400 and 800 nM [^{3}H]noradrenaline, respectively. Values represent reserpine-sensitive uptake (n = 3, ± SD).

only observed when $G\alpha o_2$ was added in either the GTPγS- or the AlF_4^--activated form. None of the other Gα-subunits was able to inhibit noradrenaline uptake (Figure 2B and C). Addition of non-activated (Figure 2B) or heat-denatured $G\alpha o_2$ (Figure 2C) had no effect. As expected, pretreatment of the cells with pertussis toxin did not prevent inhibition by purified $G\alpha o_2$ (Figure 2C), since under these conditions only endogenous Gi/o proteins would be affected.

For further characterization, down-regulation of noradrenaline uptake by GTPγS and purified $G\alpha o_2$ was analysed at different time points and at different noradrenaline concentrations (Figure 3). GTPγS- and $G\alpha o_2$-dependent reduction of reserpine-sensitive noradrenaline uptake was evident throughout the incubation period (Figure 3A). Furthermore, down-regulation appears to be due to a decrease in substrate affinity since increasing concentra-

Table I. Temperature-dependence of G protein-mediated down-regulation of monoamine transport

	NA uptake (pmol/mg protein)	
	25°C	36°C
Control	26.2 ± 0.8	32.7 ± 1.2
Reserpine	15.2 ± 3.4	15.7 ± 1.0
GTPγS	20.7 ± 2.9	26.8 ± 2.8
Gαo$_2$	17.0 ± 1.6	24.1 ± 0.7

PC 12 cells were permeabilized on ice and then further processed either by 25°C or by 36°C incubation. Values represent the mean of three samples ± SD.

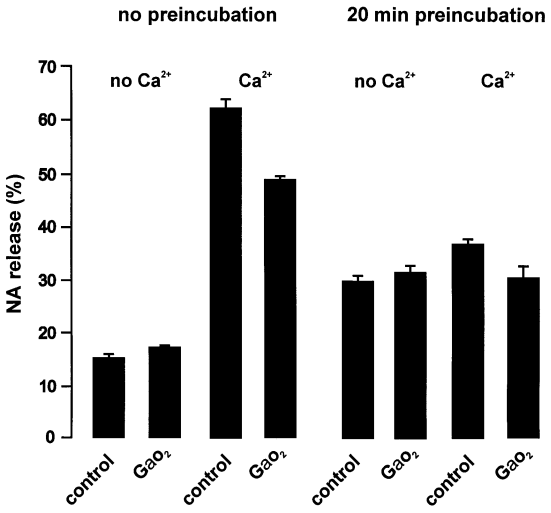

Fig. 4. Gαo$_2$ has no effect on basal noradrenaline release but inhibits Ca^{2+}-stimulated exocytosis. Preloaded PC 12 cells were permeabilized with SLO in KG-buffer and then either immediately stimulated for 10 min with KG-buffer plus ATP containing no or 15 μM free Ca^{2+} (no preincubation) or were preincubated for 20 min in KG before stimulation (20 min preincubation). 15 nM AlF_4^--activated Gαo$_2$ was added to the stimulation solution. Noradrenaline release was determined in the supernatant and the cell lysate and was given as percent of total noradrenaline content present at the beginning of stimulation (n = 3, ± SD). The increase in % basal release after preincubation is due to the loss of total [^{3}H]noradrenaline content (54 250 ± 3591 d.p.m. versus 38 139 ± 3188 d.p.m; n = 12, ± SD) rather than to an increase in basal release.

tions of noradrenaline partially compensated the inhibition (Figure 3B). Decreasing the temperature to 25°C reduced total noradrenaline uptake but the down-regulation of transport activity by both GTPγS and Gαo$_2$ was not affected (Table I).

In order to rule out that the observed reduction of uptake is due to loss of noradrenaline by exocytosis, two independent control experiments were performed. First, we studied the influence of Gαo$_2$ on basal and Ca^{2+}-stimulated exocytosis. Gαo$_2$ did not affect basal catecholamine release but inhibited Ca^{2+}-stimulated secretion (Figure 4). This observation extends a previous report (Vitale *et al.*, 1994) that demonstrated inhibition of exocytosis by Go proteins in adrenal chromaffin cells. Furthermore, Ca^{2+}-stimulated exocytosis drastically declined when permeabilized cells were preincubated before stimulation due to the loss of proteins required for secretion (Hay and Martin, 1992). Since uptake was measured after preincubation, exocytotic events did not

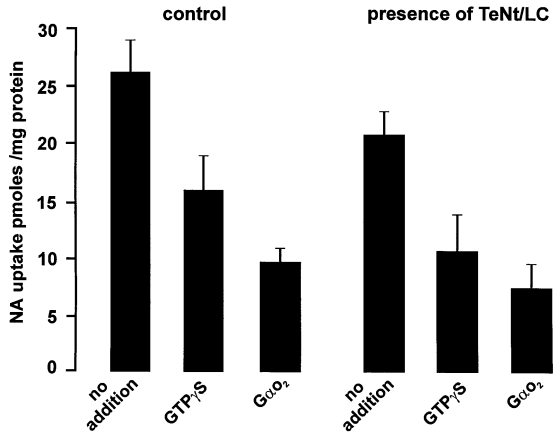

Fig. 5. Down-regulation of noradrenaline uptake is not affected when exocytosis is inhibited by tetanus toxin light chain. Permeabilized PC 12 cells were preincubated for 20 min with KG-buffer in the absence or presence of TeNt/LC (115 nM final concentration) before uptake was started by addition of fresh KG-buffer plus MgATP supplemented with 50 μM GTPγS or 15 nM AlF_4^--activated Gαo$_2$. Noradrenaline uptake was determined after 20 min of incubation. Unspecific accumulation (pmol/mg protein) in the presence of 2 μM reserpine was 14.8 ± 0.3 and 15 ± 0.2 for untreated or TeNt/LC-treated samples, respectively. Values (n = 3, ± SD) represent reserpine-sensitive uptake.

attribute to the observed inhibition of uptake. Second, permeabilized PC 12 cells were incubated with the light chain of tetanus toxin (TeNt/LC) that completely blocks exocytosis elicited by Ca^{2+} (Ahnert-Hilger *et al.*, 1990) or GTPγS (G.Ahnert-Hilger, unpublished observations). As shown in Figure 5, GTPγS- or Gαo$_2$-induced down-regulation of transport activity remained unaffected.

G protein-mediated down-regulation is due to a direct control of the monoamine transporter

The data presented so far demonstrate that activation of a pertussis toxin-sensitive Go protein, most likely Gαo$_2$, inhibits the vesicular monoamine uptake system. However, they do not distinguish between a direct inhibition of the transporter by the G protein, and an indirect inhibition of uptake mediated, for instance, by a reduction in the driving force for uptake, i.e. the electrochemical proton gradient. Two independent approaches were therefore used to discern whether G protein-mediated regulation affects the proton gradient or the transporter.

First, we examined whether ATP-dependent acidification was affected by GTP-analogues and AlF_4^-, using the pH-sensitive dye acridine orange to report changes in the pH-gradient across the vesicle membrane. Although both components of the electrochemical proton gradient, the membrane potential and the pH gradient (ΔpH), can energize catecholamine uptake, ΔpH provides the dominant driving force, particularly when the external chloride concentration is high as in our experiments (Johnson, 1988; Maycox *et al.*, 1990; Liu and Edwards, 1997). When ATP-dependent acidification was measured in permeabilized PC 12 cells under conditions similar to those used for down-regulation of transport, no difference in the rate of acidification was observed in the presence of non-hydrolysable GTP-analogues and AlF_4^-, regardless of whether the chloride concentration was low or high. Bafilomycin, a specific inhibitor of the vacuolar ATPase,

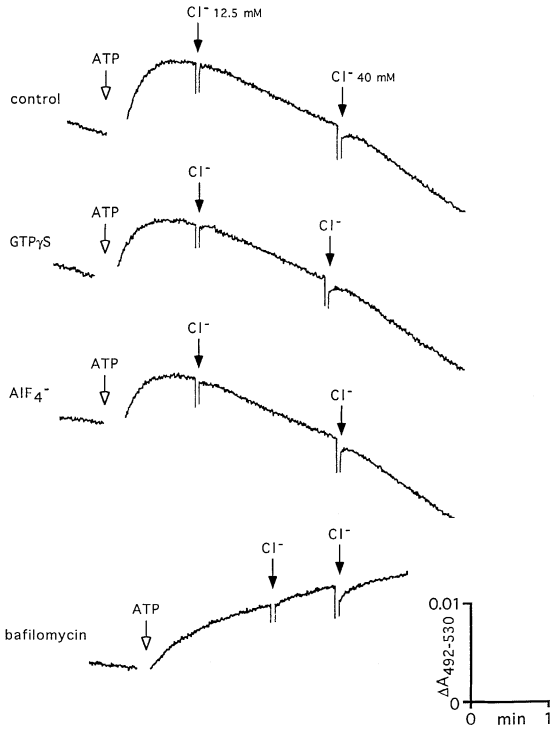

Fig. 6. Activators of heterotrimeric G proteins have no effects on ATP-dependent, bafilomycin-sensitive acidification of internal organelles in permeabilized PC 12 cells. Acidification of intracellular organelles was monitored as a change of absorbance using acridine orange as an indicator dye (dual wavelength mode). After a stable baseline was reached following addition of ATP, the rate of acidification increased when chloride ions were added, in agreement with previous results on isolated secretory organelles (Hell *et al.*, 1990). NH_4Cl reverted the signal due to a neutralization of the intravesicular lumen (not shown; Hell *et al.*, 1990). In the presence of bafilomycin, an inhibitor of vacuolar ATPase, a linear increase rather than a decrease of absorbance was observed which was not affected by chloride additions and which is probably caused by residual mitochondrial activity.

prevented acidification, confirming that our signal was due to the activity of this enzyme (Figure 6) and not due to interference by mitochondria.

As a second independent approach, we examined whether binding of reserpine to the monoamine transporter was affected under conditions of G protein-mediated down-regulation. Reserpine acts as a competitive inhibitor of the transporter and its binding is accelerated in the presence of a pH gradient (Rudnick *et al.*, 1990; Edwards, 1992; Yelin and Schuldiner, 1995; Liu and Edwards, 1997). Using [^{3}H]reserpine as a ligand, we measured specific binding in a range between 100 and 250 fmol reserpine/mg protein, depending on the batch of PC 12 cells. Binding of [^{3}H]reserpine was inhibited by addition of 2 μM non-labelled reserpine, demonstrating that binding was saturable (Figure 7A). Reserpine-sensitive binding was diminished by 50 μM GTPγS (Figure 7A and B). Preincubating the cells with pertussis toxin overcame GTPγS-induced inhibition (Figure 7B) while cholera toxin was ineffective (not shown). Addition of 15 nM AlF_4^--activated $G\alpha o_2$ mimicked the inhibition by GTPγS (Figure 7C). Together, these data demonstrate that $G\alpha o_2$ acts on the transporter and not on the energy gradient necessary for sustaining catecholamine transport across the vesicle membrane.

Discussion

In the present study, we have demonstrated that the monoamine transporter of secretory granules is regulated by the α-subunit of a specific heterotrimeric G protein, Go_2. To our knowledge, this is the first time that regulation of a vesicular neurotransmitter transporter by means other than changing the energy gradient has been reported. Furthermore, our findings document that heterotrimeric G proteins are involved in the regulation of intracellular transport processes, adding novel targets to the list of effectors for these versatile molecular switches. Using dibutyryl cyclic AMP, a modulation of monoamine transport in PC 12 cells has been shown. Since these effects were only observed when dibutyryl cAMP was given before permeabilization it remains unclear whether changes in uptake activity or in the metabolism of catecholamines were responsible (Nakanishi *et al.*, 1995). It is plausible to assume that other vesicular transporters such as the acetylcholine transporter or the transport activities for glutamate and GABA/glycine are also regulated by local interaction with heterotrimeric G proteins such as Gi or Go proteins associated with secretory and synaptic vesicles (Ahnert-Hilger *et al.*, 1994).

Go proteins comprise ~1% of the total protein of neurones and neuroendocrine cells, but knowledge concerning their specific function is scarce. Studies with knock-out mice revealed that Gαo is necessary for muscarinic regulation of calcium channels in heart (Valenzuela *et al.*, 1997). In neuroendocrine cells, it was shown that Go proteins regulate voltage-dependent calcium channels (Kalkbrenner *et al.*, 1996). Recent studies suggest that the Gβγ-complex rather than the Goα-subunit may be the active moiety in this interaction (Herlitze *et al.*, 1996; Ikeda, 1996). Although both Go and Gi proteins couple to receptors regulating calcium channel activity, only Gβγ released from Go appears to interact with calcium channels, leaving the Gαo-subunit without a specific function.

Here we present for the first time a specific interaction of an α-subunit of a Go protein with an effector system on intracellular membranes. $G\alpha o_2$ is selectively concentrated on the membrane of chromaffin granules (Ahnert-Hilger *et al.*, 1994), and Go and Gi proteins are associated with synaptic vesicles (Aronin and DiFiglia, 1992; Ahnert-Hilger *et al.*, 1994), thus being ideally positioned for controlling vesicular neurotransmitter uptake. It remains to be established whether transport activity is controlled by direct interaction of the G protein with the transporter or whether intermediate proteins are involved for signal transduction. G protein-mediated down-regulation of noradrenaline transport was largely lost when secretory vesicles were purified from bovine adrenal medulla, preventing further characterization of the underlying mechanisms. We can exclude, however, that the reduction in noradrenaline uptake (i.e. reduction of labelled transmitter in the washed cell pellet) simply reflects loss of transmitter due to exocytotic release. The light chain of tetanus toxin which cleaves synaptobrevin, thereby inhibiting exocytosis (Ahnert-Hilger *et al.*, 1990; Chilcote *et al.*, 1995), had no effects on G protein-mediated down-regulation of transmitter uptake. Furthermore, none of the purified Gαo-subunits elicited exocytosis, neither in the absence nor the

presence of stimulatory concentrations of Ca^{2+}. Rather, Ca^{2+}-dependent release was inhibited while leaving basal release unaffected, extending previous findings obtained for bovine chromaffin cells (Vitale *et al.*, 1994).

The factors responsible for the G protein-mediated control of the monoamine transporter are not known and we can speculate about the factors which might be involved in the upstream regulation of Gαo$_2$. Interestingly, a constitutive phosphorylation of the vesicular monoamine transporter VMAT2 but not VMAT1 has been reported, suggesting differential regulation of the two monoamine transporter isoforms (Krantz *et al.*, 1997). In a variety of physiologically relevant circumstances it may be highly advantageous for a neurone or neuroendocrine cell to be able to regulate vesicular uptake. Two opposite scenarios may give a physiological relevance for a G protein-mediated down-regulation. For instance, it is possible that a sensor in the vesicle interior reports the degree of neurotransmitter loading and shuts the system off when the vesicle is full. Since we looked at a heterogeneous population of vesicles this would result in the partial reduction of the overall uptake. Secondly, monoamine transporters are characterized by high affinities for their substrates which are quite different from the considerably lower values of vesicular acetylcholine transporter and the transporters for GABA/glycine and glutamate (Liu and Edwards, 1997). Thus, decreasing the affinity of the transporter for monoamines by Go may enable the cell to get rid of high amounts of probably toxic monoamines in the cytoplasm and rapidly to refill secretory vesicles for another cycle. An attractive feature of this scenario is that signal transduction would involve transmembrane signalling similar to the coupling of heterotrimeric G proteins to transmembrane receptors (Nürnberg and Ahnert-Hilger, 1996). However, it is equally possible that uptake is down-regulated during membrane biogenesis or endocytic processing, which means that the transporter is regulated by factors controlling the membrane traffic of secretory vesicles. In this context, it is interesting to note that Gαo has also been implicated as a negative regulator of Ca^{2+}-dependent exocytosis in chromaffin cells (Vitale *et al.*, 1994) and PC 12 cells (this paper). Thus, as a third possibility Gαo$_2$ may be activated by cytoplasmic factors that down-regulate both exocytotic release and uptake in parallel.

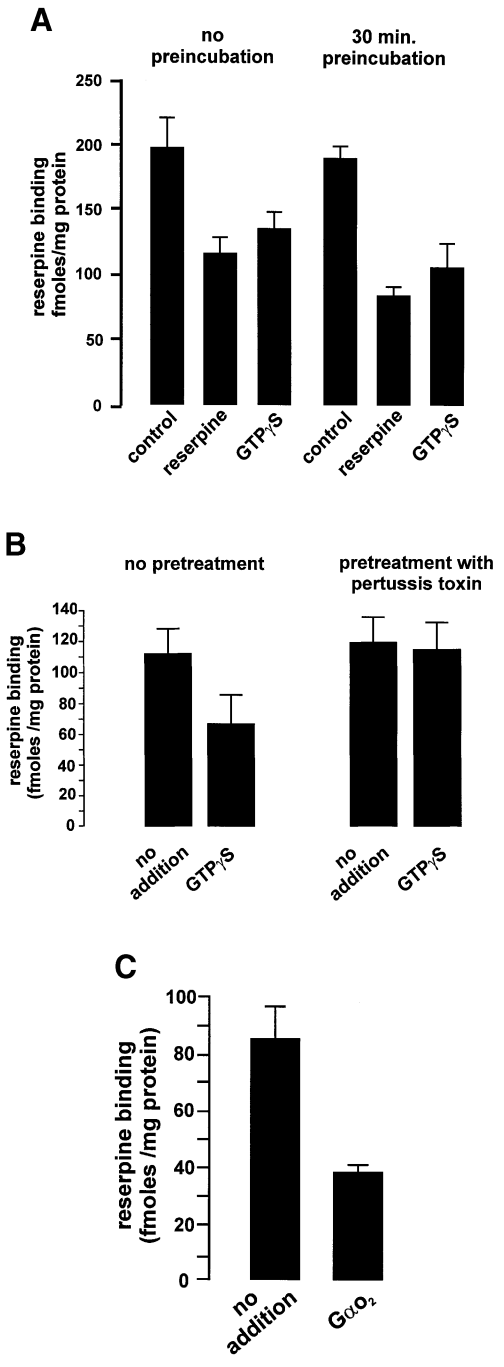

Fig. 7. Pertussis toxin-sensitive Go$_2$ inhibits reserpine binding in permeabilized PC 12 cells. (**A**) SLO-permeabilized PC 12 cells were resuspended at 4°C in KG-buffer plus MgATP containing no additives (control), 2 μM unlabelled reserpine (reserpine) or 50 μM GTPγS. 1 μl of 200 nM [^{3}H]reserpine was added given a final concentration of 2 nM and samples were incubated for 5 min at 36°C before the reaction was stopped by addition of ice-cold KG-buffer (no preincubation). A second set of samples were preincubated in KG-buffer plus ATP with 2 μM unlabelled reserpine or 50 μM GTPγS for 30 min at 36°C before [^{3}H]reserpine was added and incubated for another 5 min. Binding of [^{3}H]reserpine in the absence of membranes was 3.3 ± 0.2 fmol/tube, corresponding to ~2% of total radioactivity per tube and was subtracted from each sample. (**B**) Addition of GTPγS (50 μM final concentration) inhibited specific reserpine binding. After incubation with SLO cells were resuspended in KG-buffer containing MgATP and either no additives (control) or 50 μM GTPγS. After 10 min at 36°C binding was started by the addition of 1 μl 200 nM [^{3}H]reserpine and incubation continued for 10 min. Inhibition was prevented by preincubating the cells with pertussis toxin. Unspecific binding (fmol/mg protein) in the presence of 2 μM unlabelled reserpine was 170 ± 20 and 200 ± 50 for untreated or pertussis toxin-treated samples, respectively. (**C**) Purified AlF_4^--activated Gαo$_2$ (15 nM final concentration) inhibited specific binding of reserpine. The experimental design was the same as in Figure 2B. Unspecific binding in the presence of 2 μM unlabelled reserpine was 70 ± 8 fmol/mg protein. Values (n = 3, ± SD) in (B) and (C) are corrected for unspecific binding obtained in the presence of 2 μM unlabelled reserpine.

Materials and methods

Catecholamine uptake

PC 12 cells were grown as described (Ahnert-Hilger *et al.*, 1989a). The medium was removed and cells were washed three times with phosphate-buffered saline (PBS) before they were suspended in potassium sucrose buffer (KS-buffer) containing 50 mM KCl, 200 mM sucrose, 20 mM PIPES, 4 mM EGTA, 1 mM $MgCl_2$, adjusted to pH 7.0 with KOH. Cells were incubated with SLO (Weller *et al.*, 1996) dissolved in uptake

buffer for 10 min on ice (Ahnert-Hilger *et al.*, 1989b). The buffer was replaced with 100 µl KS-buffer and the cells were incubated for 20 min at 36°C to remove endogenous ATP. The buffer was then removed and the cells were incubated with 100 µl KS-buffer containing 0.5 µCi [^{3}H]noradrenaline (15 Ci/mmol), 2 mM MgATP, 2 mM reserpine, GTP-analogues, or purified G protein α-subunits (as indicated) for 15–20 min at 36°C. The incubation was stopped by addition of 1 ml of ice-cold KS-buffer followed by rapid centrifugation of the cells. The supernatant was removed and the cell pellet washed once with 500 µl ice-cold KS-buffer. The cell pellet was lysed in 0.4% Triton X-100 to determine radioactivity by scintillation counting and protein content using the bicinchoninic acid method (BCA-Kit; Pierce). For some experiments, potassium glutamate buffer (KG-buffer) containing 150 mM potassium glutamate, 20 mM PIPES, 4 mM EGTA, 1 mM $MgCl_2$, adjusted to pH 7.0 with KOH, supplemented with 2 mM MgATP and substances to be tested as described for KS-buffer. There was no difference in the ATP-dependent and reserpine-sensitive uptake when KG-buffer instead was used.

Incubation of cells with pertussis toxin (100 ng/ml) was performed overnight. In another series of experiments, the cell pellets, following incubation with SLO were resuspended in 20 µl uptake buffer in the presence or absence of an affinity-purified antiserum (AS 6) specific for Gαo-subunits (Spicher *et al.*, 1992). The cells were preincubated for 10 min at 36°C before the addition of 100 µl uptake buffer supplemented with 0.5 µCi [^{3}H]noradrenaline and 2 mM MgATP. Incubation and analysis was then performed as described above.

Catecholamine secretion

Measurement of catecholamine secretion was performed as described elsewhere (Ahnert-Hilger *et al.*, 1989b, 1992). Briefly, cells were preloaded with [^{3}H]noradrenaline, washed and permeabilized by SLO dissolved in KG-buffer. They were either stimulated directly in KG-buffer supplemented with 2 mM ATP or first preincubated in KG-buffer plus ATP in the presence of the Gαo-subunit before they were stimulated. Noradrenaline was measured in the supernatant and in the cells after dissolving them in Triton X-100 (0.4%). Release is given as the percent of noradrenaline content present at the beginning of stimulation.

Acidification of internal organelles

PC 12 cells were washed three times with PBS, suspended in PBS and stored as pellets of ~1 mg cell protein on ice. Cells were resuspended in acidification buffer containing 300 mM sucrose, 2 mM $MgSO_4$, 2 mM $MgCl_2$, 10 mM MOPS, adjusted to pH 7.4 with KOH and supplemented with SLO as above. After a 10 min incubation on ice, the buffer was removed and the cells were resuspended in acidification buffer supplemented with 200 µM ouabain, 100 µM sodium vanadate, 10 µM oligomycin B, 20 µM acridine orange, 68 µg/ml creatine kinase and 13.6 mM creatine phosphate and incubated at 32°C. Acidification was monitored as decrease of absorbance of the pH-sensitive dye acridine orange at 492 nm with 530 nm as a reference in a 1 ml cuvette using a SLM-Aminco DW-2000 double beam spectrophotometer, as detailed elsewhere (Hell *et al.*, 1990). G protein-activators or bafilomycin were added as indicated before registration was started, followed by the addition of 4.8 mM ATP and KCl, respectively.

Purification of Gα isoforms

Pertussis toxin-sensitive Gα-isoforms were purified from bovine brain as described elsewhere (Nürnberg *et al.*, 1994). The heterotrimeric G proteins were dissociated into their subunits by AlF_4^- and the Gα- and Gβγ-subunits and separated by hydrophobic interaction chromatography with heptylamine–Sepharose. The various Gαi- and Gαo-isoforms were separated using an anion exchange chromatography (FPLC/Mono Q) and identified by Western blotting using subtype-specific antibodies. Before experiments, purified Gα-subunits were diluted 1:100 with 1 mM EDTA, 10 mM NaCl, 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, 20 mM Tris–HCl, pH 8, and concentrated (Nürnberg *et al.*, 1994). In some experiments Gα-isoforms were either activated by GTPγS (10 µM) diluted 10- to 100-fold before the experiment or applied in their inactive GDP-bound form. Control experiments revealed that the residual amount of GTPγS (100 nM final concentration) carried over into the uptake experiments did not cause any change in uptake activity.

[^{3}H]Reserpine binding

PC 12 cells were washed three times with PBS before they were suspended in KG-buffer. The buffer was replaced by fresh KG-buffer containing SLO and cells were incubated for 10 min on ice. The buffer was replaced by 100 µl KG-buffer supplemented with 2 mM MgATP and substances to be tested. The cells were incubated for 10 min at 36°C before the addition of 1 µl [^{3}H]reserpine (200 nM) resulting in a final concentration of 2 nM. Incubation was continued for another 5–10 min at 36°C before being stopped by addition of 1 ml of ice-cold KG-buffer followed by rapid centrifugation of the cells. The supernatant was removed and the cell pellet washed once with 500 µl ice-cold KG-buffer. The cell pellet was lysed in 0.4% Triton X-100 to determine radioactivity by scintillation counting and protein content. Unspecific binding was determined in the presence of 2 µM unlabelled reserpine (Rudnick *et al.*, 1990; Stern-Bach *et al.*, 1990; Peter *et al.*, 1994). In the reserpine binding studies KG-buffer was superior to the KS-buffer used for some of the uptake studies.

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References

Ahnert-Hilger,G., Bräutigam,M. and Gratzl,M. (1989a) Ca^{2+}-stimulated catecholamine release from permeabilized PC 12 cells: biochemical evidence for exocytosis and its modulation by protein kinase C and G-proteins. *Biochemistry*, **26**, 7842–7848.

Ahnert-Hilger,G., Mach,W., Föhr,K.-J. and Gratzl,M. (1989b) Poration by alphatoxin and streptolysin O: an approach to analyze intracellular processes. *Methods Cell Biol.*, **31**, 63–90.

Ahnert-Hilger,G., Dauzenroth,M.-E., Habermann,E., Henschen,A., Krieglstein,K., Mauler,F. and Weller,U. (1990) Chains and fragments of tetanus toxin and their contribution to toxicity. *J. Physiol. Paris*, **84**, 229–236.

Ahnert-Hilger,G., Wegenhorst,U., Stecher,B., Spicher,K., Rosenthal,W. and Gratzl,M. (1992) Exocytosis from permeabilized bovine adrenal chromaffin cells is differently modulated by GTPτS and GMPPNHP. Evidence for the involvement of various guanine nucleotide binding-protein. *Biochem. J.*, **284**, 321–326.

Ahnert-Hilger,G., Schäfer,T., Spicher,K., Grund,C., Schultz,G. and Wieden-mann,B. (1994) Detection of G-protein heterotrimers on large dense core and small synaptic vesicles of neuroendocrine and neuronal cells. *Eur. J. Cell Biol.*, **65**, 26–28.

Aronin,N. and DiFiglia,M. (1992) The subcellular localization of G-protein $G_i\alpha$ in the basal ganglia reveals its potential role in both signal transduction and vesicle trafficking. *J. Neurosci.*, **12**, 3435–3444.

Bhakdi,S. and Tranum-Jensen,J. (1987) Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 147–223.

Charbre,M. (1990) Aluminofluoride and berylofluoride complexes: a new phosphate analog in enzymology. *Trends Biochem. Sci.*, **15**, 6–10.

Chilcote,T.J., Galli,T., Mundigl,O., Edelmann,L., McPherson,P.S., Takai,K. and DeCamilli,P. (1995) Cellubrevin and synaptobrevins: similar subcellular localization and biochemical properties in PC 12 cells. *J. Cell Biol.*, **129**, 219–231.

Edwards,R.H. (1992) The transport of neurotransmitters into synaptic vesicles. *Curr. Opin. Neurobiol.*, **2**, 586–594.

Hay,J.C. and Martin,T.F.J. (1992) Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. *J. Cell Biol.*, **119**, 139–151.

Hell,J.W., Maycox,P.R. and Jahn,R.J. (1990) Energy dependence and functional reconstitution of the γ-aminobutyric acid carrier from synaptic vesicles. *Biol. Chem.*, **265**, 2111–2117.

Herlitze,S. Garcia,D.E., Mackie,K., Hille,B., Scheuer,T. and Catterall,W. (1996) Modulation of Ca^{2+} channels by G-protein βγ subunits. *Nature*, **380**, 258–262. (Addendum, *Nature*, **381**, 172.)

Ikeda,S.R. (1996) Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. *Nature*, **380**, 255–258.

Jakobs,K.H., Aktories,K. and Schultz,G. (1984) Mechanism of pertussis toxin action on the adenylate system: inhibition of the turn-on reaction of the inhibitory regulatory site. *Eur. J. Biochem.*, **140**, 177–181.

Johnson,R. (1988) Accumulation of biological amines into chromaffin granules: a model for hormone and neurotransmitter transport. *Physiol. Rev.*, **68**, 232–307.

Kalkbrenner,F., Dippel,E., Wittig,B. and Schultz,G. (1996) Specificity of interaction between receptor and G-protein: use of antisense techniques to relate G-protein subunits to function. *Biochim. Biophys. Acta*, **1314**, 125–139.

Krantz,D.E., Peter,D., Liu,Y. and Edwards,H. (1997) Phosphorylation of a vesicular monoamine transporter by casein kinase II. *J. Biol. Chem.*, **272**, 6752–6759.

Krejci,E., Gasnier,B., Botton,D., Sambert,M.F., Sagne,C., Gagnon,J., Massoulie,J. and Henry,J.P. (1993) Expression and regulation of the bovine vesicular monoamine transporter gene. *FEBS Lett.*, **335**, 227–232.

Liu,Y. and Edwards,R. (1997) The role of vesicular transport proteins in synaptic transmission and neural degeneration. *Annu. Rev. Neurosci.*, **20**, 125–156.

Liu,Y., Peter,A., Roghani,A., Schuldiner,S., Prive,G.G., Eisenberg,D., Brecha,N. and Edwards,R. (1992) A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. *Cell*, **70**, 539–551.

Maycox,P.R., Hell,J.W. and Jahn,R. (1990) Amino acid neurotransmission: spotlights on synaptic vesicles. *Trends Neurosci.*, **13**, 83–87.

Nakanishi,N., Onozawa,S., Matsumoto,R., Hasegawa,H. and Yamada,S. (1995) Cyclic AMP-dependent modulation of vesicular monoamine transport in pheochromocytoma cells. *J. Neurochem.*, **64**, 600–607.

Nürnberg,B. and Ahnert-Hilger,G. (1996) Potential roles of heterotrimeric G proteins of the endomembrane system. *FEBS Lett.*, **389**, 61–65.

Nürnberg,B., Spicher,K., Harhammer,R., Bosserhoff,A., Frank,R., Hilz,H. and Schultz,G. (1994) Purification of a novel G-protein α_o-subtype from mammalian brain. *Biochem. J.*, **300**, 387–394.

Peter,D., Jiminez,J., Liu,Y., Kim,J. and Edwards,R.H. (1994) The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. *J. Biol. Chem.*, **269**, 7231–7237.

Rudnick,G., Steiner-Mordoch,S.S., Fishkes,H., Stern-Bach,Y. and Schuldiner,S. (1990) Energetics of reserpine binding and occlusion by the chromaffin granule biogenic amine transporter. *Biochemistry*, **29**, 603–608.

Schloss,P., Püschel,A.W. and Betz,H. (1994) Neurotransmitter transporters: new members of known families. *Curr. Opin. Cell Biol.*, **6**, 595–599.

Schuldiner,S., Fishkes,H. and Kanner,B.I. (1978) Role of a transmembrane pH gradient in epinephrine transport by chromaffin granule membrane vesicles. *Proc. Natl Acad. Sci. USA*, **75**, 3713–3716.

Spicher,K., Nürnberg,B., Jäger,B., Rosenthal,W. and Schultz,G. (1992) Heterogeneity of three electrophoretically distinct G_o α-subunits in mammalian brain. *FEBS Lett.*, **307**, 215–218.

Stern-Bach,Y., Greenberg-Ofrath,N., Flechner,I. and Schuldiner,S. (1990) Identification and purification of a functional amine transporter from bovine chromaffin granules. *J. Biol. Chem.*, **265**, 3961–3966.

Stern-Bach,Y. Keen,J.N., Bejerano,M., Steiner-Mordoch,S., Wallach,M., Findlay,J.B. and Schuldiner,S. (1992) Homology of a vesicular amine transporter to a gene conferring resistance to 1-methyl-4-phenylpyridinium. *Proc. Natl Acad. Sci. USA*, **89**, 9730–9733.

Valenzuela,D., Han,X., Mende,U., Fankhauser,C., Mashimo,H., Huang,P., Pfeffer,J., Neer,E.J. and Fishman,M.C. (1997) $G\alpha_o$ is necessary for muscarinic regulation of Ca^{2+} channels in mouse heart. *Proc. Natl Acad. Sci. USA*, **94**, 1727–1732.

Van der Kloot,W. (1991) The regulation of quantal size. *Prog. Neurobiol.*, **36**, 93–130.

Vitale,N., Deloulme,J.-C., Thierse,D., Aunis,D. and Bader,M.F. (1994) GAP-43 controls the availability of secretory chromaffin granules for regulated exocytosis by stimulating a granule-associated Go. *J. Biol. Chem.*, **269**, 30293–30298.

Weller,U., Müller,L., Meβner,M. and Bhakdi,S. (1996) Expression of active streptolysin-O in *E. coli* as maltose-binding protein–streptolysin-O fusion protein: the N-terminal 70 amino acids are not required for hemolytic activity. *Eur. J. Biochem.*, **236**, 34–39.

Yelin,R. and Schuldiner,S. (1995) The pharmacological profile of the vesicular monoamine transporter resembles that of multidrug transporters. *FEBS Lett.*, **377**, 201–207.

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