How environment affects conditioned drug tolerance

Repeated drug administration in a given stimulus context may induce the development of a conditioned response, and this response may occur in anticipation of [1], and interact with [2], the regular 'unconditioned' effect of the drug. Very often, the conditioned response is a 'compensatory' response; i.e. one that consists of an effect opposite to the one that would be expected from the drug (e.g. an increase instead of a decrease of locomotor activity).

Siegel and his co-workers have expanded these early observations into a conditioned-reflex model of drug tolerance, and have indeed demonstrated a role of conditioning in the development of tolerance for the analgesic effect of morphine, and for the hypothermic effects of ethanol, among other drugs[3]. The problem is of practical as well as theoretical importance. A lot of the drug-seeking behavior of drug abusers is said to be conditioned, partly to the environment and/or the social context; and it is common for drug abusers to switch from a given drug or drug combination to another (usually cheaper or more socially acceptable) in order to procure the same 'high'. Many die in this quest, either because they succeed or because they don't.

Two papers bearing on this point have recently appeared. Palletta and Wagner[4] reported on the development of context-specific tolerance to both the analgesic and the activity-depressant effects of morphine in rats: conditioned 'compensatory' responses occurred only in the activity measure. Krank and Bennett[5] reported on the development of context-specific conditioning to the locomotor-stimulant effect of (+)-amphetamine, also in rats: pre- and post-drug activity levels increased over a number of daily sessions but only in the box where the rats received the drug. More importantly, Krank and Bennett showed that this response is transferred to morphine, even when morphine is given at a dose that normally should induce a decrease, rather than an increase, in activity[5].

The implications of these findings for human addictive behavior are obvious, particularly in relation to environment or contextual effects and drug switches.

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CURRENT TECHNIQUES

Controlled manipulation of the cell interior by pore-forming proteins

Experimental analysis of intracellular processes is hampered by the limiting plasma membrane. Thus data from intact cells often provide only indirect evidence on the nature and regulation of intracellular events. Isolated enzymes and purified subcellular organelles can be characterized biochemically but this gives no information about their cellular function.

In contrast, permeabilization of the plasma membrane by appropriate techniques allows access to, and control over, the cytosol while leaving the intracellular organization intact. Such permeabilized cell preparations permit study of the regulation of exocytosis in secretory cells, a process which is controlled by intracellular events and involves the fusion of secretory vesicles with the plasma membrane. The chain of events leading to exocytosis and its modulation are still not completely understood.

Techniques for membrane permeabilization

Exocytosis can be studied in permeabilized secretory cells, provided that neither the vesicle membrane nor the plasma membrane has been disturbed by the permeabilization procedure. Permeabilization of secretory cells has hitherto been performed by physical or chemical techniques.

High voltage discharge, first used with adrenal medullary chromaffin cells[1] allows dialysis of small molecules from the cell interior, whereas soluble cytoplasmic proteins remain within the cell[2]. Electrical permeabilization is especially suitable for cells in suspension.

The whole cell patch clamp technique, which records the increase in capacitance of the plasma membrane during exocytosis, is a method for permeabilization of single cells. It allows the introduction of not only small molecules but also high molecular weight proteins into the cell. Furthermore it is possible to analyse the fusion of single vesicles with the plasma membrane[3].

Detergents can be used to permeabilize cultured cells in suspension or attached to culture plates. In the past, saponin and digitonin have been used for the permeabilization of adrenal medullary chromaffin and other secretory cells[2,4–6]. However, detergents may destroy intracellular membranes[2,7].

To overcome these and other difficulties inherent in the previous techniques we have employed bacterial toxins for the stable, selective and prolonged permeabilization of the plasma membrane of secretory cells[7–10]. Two pore-forming proteins, α-toxin from *Staphylococcus aureus* and streptolysin O from β-hemolytic streptococci, permit the permeabilization of secretory cells

either for smaller molecules, i.e. up to 1000 Da, or for large proteins such as lactate dehydrogenase or immunoglobulins, respectively[7–12].

Since the pores formed in the membrane by the hexamerization of the α-toxin molecules are too small to allow the monomer (34 kDa) to pass[13], its attack is clearly restricted to the plasma membrane. Streptolysin O pores are large enough for the free passage of the monomer (64 kDa). However, the experimental protocol (which involves binding to the extracellular side of the plasma membrane in the cold followed by warming to 30°C to trigger pore formation) makes an attack of intracellular membranes rather unlikely[13]. In contrast, membrane permeabilization by digitonin is insensitive to the incubation temperature and therefore difficult to control (G. Ahnert-Hilger, M-F. Bader, S. Bhakdi and M. Gratzl, unpublished). The main features of permeabilization obtained by α-toxin, streptolysin O and digitonin are compared in Fig. 1.

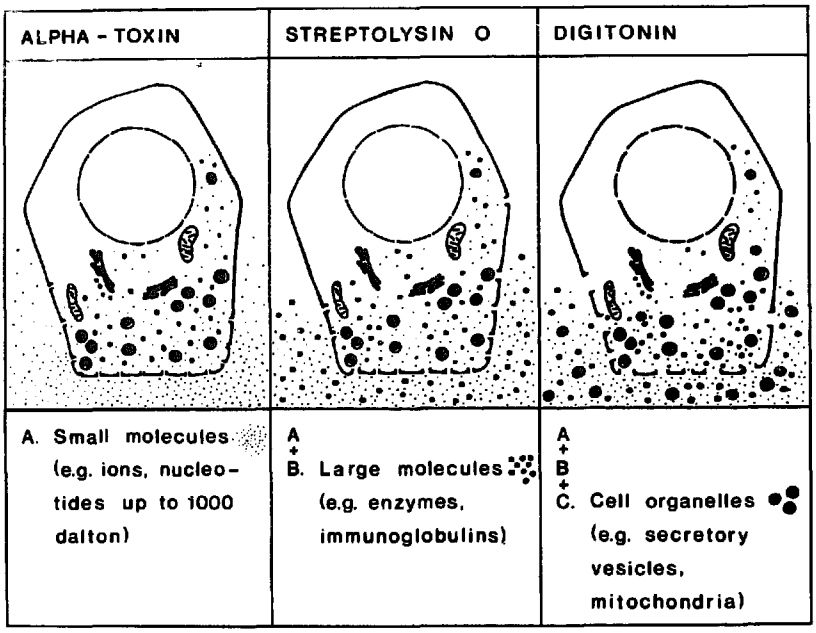

Fig. 1. Properties of cells permeabilized by the pore-forming toxins, **α-toxin** (Refs 7, 10, 11, 13 and 23) *and* **streptolysin O** (Refs 7, 12, 13, 22 and 23), *and by* **digitonin** (Refs 7 and 10).

Exocytosis by chromaffin cells

For the study of the final events during secretion by exocytosis we used the rat pheochromocytoma cell line PC 12 and adrenal medullary chromaffin cells in primary culture. Both take up catecholamines and release them on stimulation[9,14,15]. Since primary cultures of adrenal medullary chromaffin cells also contain other cell types (cortical and endothelial cells, as well as fibroblasts), the clonal cell line PC 12 is more suitable for the study of secretion since it consists of a homogeneous cell population[14,15]. α-Toxin-permeabilized cells release catecholamines by exocytosis more than an hour after toxin treatment[11], and cells permeabilized by streptolysin O do so for at least 30 min[12].

The basic properties of catecholamine release by both permeabilized chromaffin types are summarized as follows:

- The release occurs by exocytosis. This has been proven for bovine adrenal medullary cells by the parallel release of noradrenaline and the vesicular protein chromogranin A, while cytoplasmic lactate dehyrogenase is retained under these conditions[9]. Also, the lack of catecholamine metabolites (which would be formed if the transmitter had access to the cytoplasm of permeabilized PC 12 cells) strongly indicates an exocytotic event[16].
- In permeabilized PC 12 cells exocytosis is exclusively triggered by Ca^{2+} (Refs 7, 8, 11 and 16). In contrast, catecholamine release from adrenal medullary chromaffin cells requires both Ca^{2+} and Mg^{2+}/ATP[1,2,4–6,9,12]. It is probable that the random distribution of the vesicles in adrenal medullary chromaffin cells necessitates ATP-driven organelle transport during exocytosis which precedes the Ca^{2+}-induced membrane fusion[15]. Such a transport may be not required in PC 12 cells where the vesicles are preferentially located near the plasma membrane[17].

Modulation of exocytosis by permeabilized PC 12 cells

Since exocytosis in permeabilized PC 12 cells can be triggered by micromolar concentrations of Ca^{2+} alone, provided that pH and osmolarity are maintained within a physiological range, studies concerning the regulation and modulation of exocytosis are feasible. Thus the roles of Mg^{2+}, protein kinase C and GTP-binding proteins during the final steps of exocytosis have been investigated using α-toxin-permeabilized PC 12 cells.

In permeabilized PC 12 cells, free concentrations of Mg^{2+} of approximately 1 mM increase Ca^{2+}-induced exocytosis[1], whereas, in adrenal medullary chromaffin cells, Mg^{2+} alone has no effect but must be complexed to ATP to elicit exocytosis[18]. Further investigations are required to ascertain the precise role of Mg^{2+} during exocytosis.

Activation of protein kinase C by the diacylglycerol analogue OAG (1-oleyl-2-acetylglycerol) or the phorbol ester TPA (1-*O*-tetradecanoylphorbol-13-acetate) ameliorates the Ca^{2+}-induced exocytosis by both permeabilized PC 12 cells[16] and adrenal medullary chromaffin cells (G. Ahnert-Hilger, M-F. Bader, S. Bhakdi and M. Gratzl, unpublished) due to an increase in the Ca^{2+} sensitivity of the release process. In PC 12 cells[16] this effect is absolutely dependent on the presence of Mg^{2+}/ATP, while in adrenal medullary chromaffin cells the ATP dependence of protein kinase C activation cannot be clearly separated from the ATP-dependent process of exocytosis.

GTP-binding proteins (G proteins) are specialized membrane-associated proteins involved in the transduction of diverse sig-

nals[19]. Activation of G proteins by the GTP analogue GTPγS partially inhibits the Ca^{2+}-induced exocytosis by PC 12 cells in a Mg^{2+}-dependent manner. Pretreatment of the cells with pertussis toxin has no direct effect on Ca^{2+}-induced exocytosis but overcomes the observed inhibition by GTPγS. This probably indicates the regulatory control of exocytosis by PC 12 cells through a pertussis toxin-sensitive G protein[16]. Similar results have been obtained with freshly isolated bovine adrenal medullary chromaffin cells permeabilized by high voltage discharge[20]. In contrast, a stimulatory effect of GTPγS on exocytosis has been reported for chicken adrenal medullary chromaffin cells[20], bovine adrenal medullary cells in primary culture[21], mast cells[3,22] and neutrophils[22]. These contradictory results may be due to the different cell preparations used and/or reflect the regulation of exocytosis by different G proteins.

PC 12 cells permeabilized for either small or large molecules may be an ideal tool to solve some of these discrepancies and to gain more insight into the molecular events that occur during exocytosis. They are easy to handle and require only Ca^{2+} for exocytosis. Figure 2 summarizes current data concerning the regulation and modulation of exocytosis by PC 12 cells.

Further applications of permeabilized cells

The unique role of Ca^{2+} as the promoter of exocytosis is well established, but little is known of the exact mechanisms involved. In chromaffin cells there is evidence that exocytosis is not a calmodulin-dependent process since antagonists and antibodies against calmodulin are without effect on either streptolysin O – (G. Ahnert-Hilger, M-F. Bader, G. Bhakdi and M. Gratzl, unpublished) or α-toxin-permeabilized[11] cells. Application of antibodies against intracellular proteins through the well defined streptolysin O pores can help in the search for key proteins involved in exocytotic membrane fusion.

However, the use of cell permeabilization is not restricted to the study of exocytosis. Intracellular Ca^{2+} movements can be studied giving a more precise picture of Ca^{2+} regulation during exocytosis and Ca^{2+} sequestration within secretory cells.

Questions concerning compartmentalization, biosynthesis and metabolism of catecholamines can also be addressed using permeabilized cells[16]. Furthermore, the intracellular handling of fuels like glucose[23] or the action of drugs and their metabolism may easily be studied with these cells.

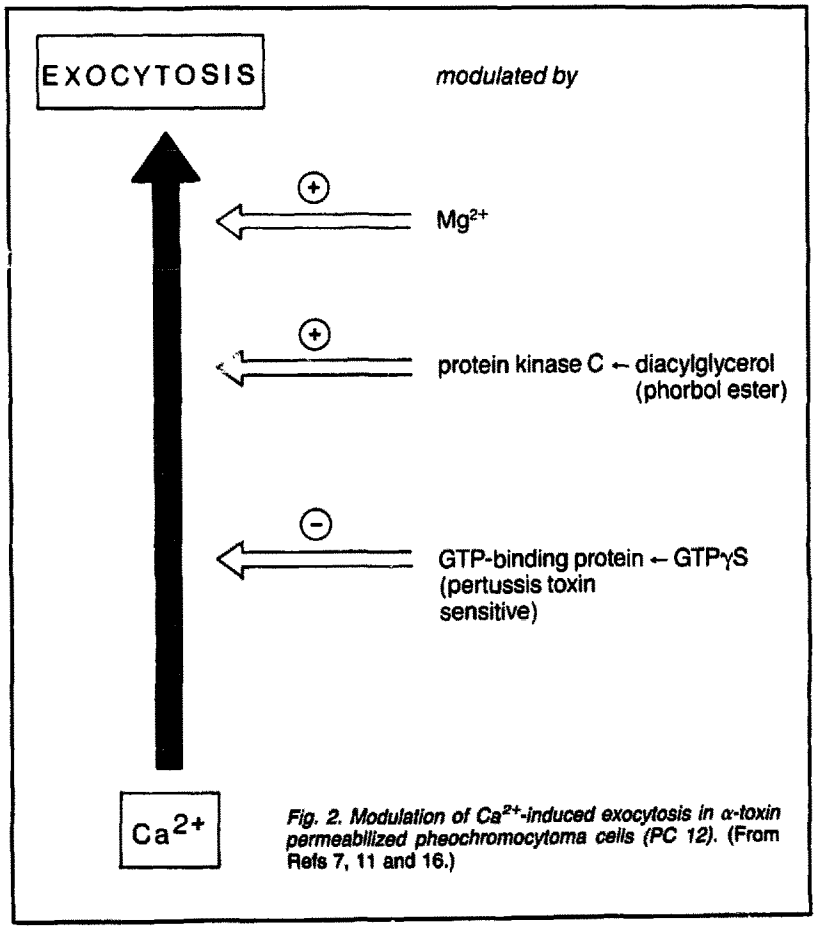

Fig. 2. Modulation of Ca^{2+}-induced exocytosis in α-toxin permeabilized pheochromocytoma cells (PC 12). (From Refs 7, 11 and 16.)

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