

T Cell Signaling and Activation: No Simple Matter

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T cells are central players of the adaptive immune response, which help protect the host against different pathogens ranging from bacteria to fungi and viruses. In order to perform their function, T cells need to be activated, a process that could lead to a variety of responses including proliferation, migration, cytokine production and even apoptosis. The “decision” by T cells to become activated or not is crucial: an inappropriate or exaggerated response could lead to autoimmune diseases while a failure to respond could lead to infection and death. To perform such a complex and sensitive task, T cells must respond to environmental cues that stimulate a complex signaling cascade. In the last few years, the signaling mechanisms that govern T cell activation and their subsequent cellular responses have been closely studied. In the present review we will examine the main signaling cascades involved and discuss several molecules that are being used to specifically block some of these pathways.

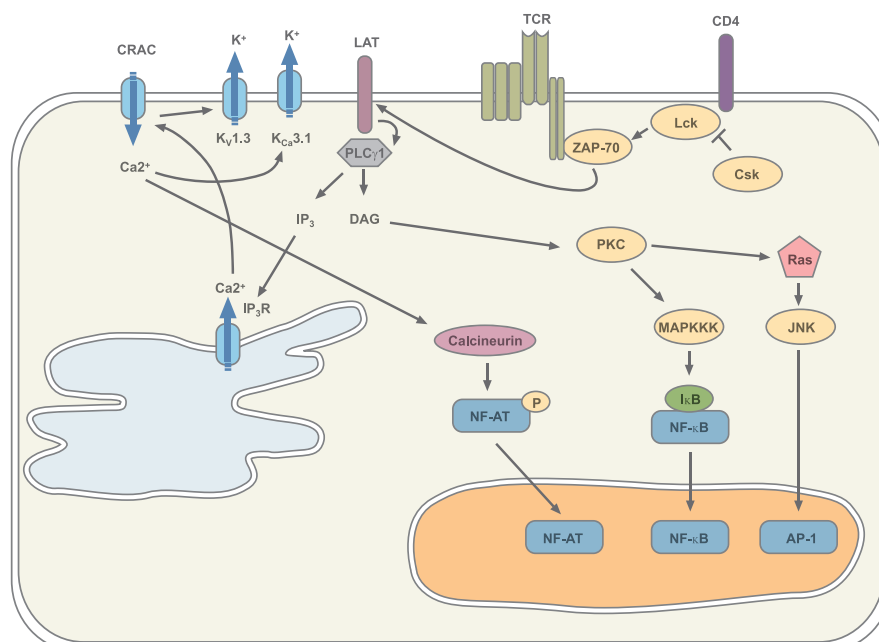
Introduction

T cell activation that leads to a productive response (i.e. cytotoxicity of target cells or stimulation of antibody production by B cells) needs two signals. The primary signal (or signal one) is provided by the binding of foreign antigens (usually small peptides from the respective pathogen) to the T cell antigen receptor (TCR) in the context of the class II major histocompatibility complex (MHC). The TCR is a complex of six different polypeptides. The clonotypic α and β chains provide the specificity of the ligand binding by a process of genetic rearrangement that provides millions of receptor variants. While the α and β heterodimer binds directly to the peptide/MHC complex, the engagement of the intracellular signaling machinery is through the invariant components of the TCR: the γ , δ and ϵ chains (collectively known as the CD3 complex) and the ζ chains.^{1,2}

Signal two or the costimulatory signal is provided by interaction of coreceptors such as CD28 or CD4 with their counterparts in the antigen presenting cell (APC).^{1,2}

The main signaling pathways elicited by binding of the TCR and some of the coreceptors are depicted in Figure 1.

Fig. 1: Major Signaling Pathways in T Cell Activation



Please note that not all the molecules involved in the signaling cascades are illustrated here for simplification purposes.

The Tyrosine Kinases (and their kinases) Signaling Pathway

The principal signaling pathway following TCR engagement is protein tyrosine phosphorylation. The tyrosine phosphorylation cascade will ultimately activate several transcription factors such as NF-AT and AP-1 which in turn direct the transcription of the new genes needed for T cell response.

The earliest detectable event following ligation of the TCR by the peptide-MHC complex is the activation of the Src family protein tyrosine kinases Lck and Fyn.³ They phosphorylate specific motifs (called immunoreceptor tyrosine-based activation motifs or ITAMs) present on the ζ and CD3 subunits of the TCR complex. Phosphorylation of these motifs promotes recruitment and activation of ZAP-70, another tyrosine kinase which in turn activates several target proteins including the adaptor/linker proteins LAT and SLP-76.^{4,5,6} Phosphorylation of LAT and SLP-76 recruits into their vicinity and facilitates the activation by tyrosine phosphorylation of the phospholipase $Cy1$ (PLC γ 1). PLC γ 1 catalyzes the formation of the second messengers, inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG), which, respectively, trigger Ca²⁺ flux and contribute to protein kinase C (PKC) and Ras activation (see also below).⁷

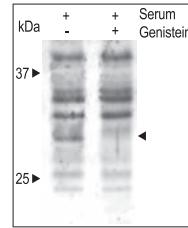
Since protein tyrosine phosphorylation is such a crucial force in T cell signaling it must be carefully controlled. This is achieved mainly by the action of the tyrosine kinase Csk which phosphorylates a critical Tyr at the C-terminal domain of Lck rendering it inactive.³

The preponderancy of the tyrosine kinase cascade in T cell signaling is demonstrated by the use of tyrosine kinase inhibitors such as **Genistein** which are able to block T cell function both *in vitro* and *in vivo*.^{8,9} Other non-specific kinase inhibitors such as **Staurosporine** can also be used to block T cell function.

PKC and Ras activation results in the activation of several members of the mitogen-activated protein kinase (MAPK) superfamily. The MAPKs are serine/threonine kinases that activate kinases (and other) signaling cascades that result in the activation of the transcription factors NF- κ B and AP-1, both of which are necessary for the transcription of several key genes involved in the T cell immune response. Three MAPK subfamilies have been described: the extracellular signal-regulated kinase (ERK), the Jun-NH2-terminal kinase (JNK) and the p38, all of which play an important role in T cell activation.

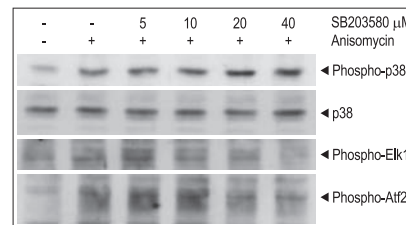
Use of specific inhibitors for the different MAPK such as the p38 inhibitor **SB203580** or the ERK inhibitor **U0126** have helped unravel the complex

Genistein: A Tyrosine Kinase and Topoisomerase Inhibitor



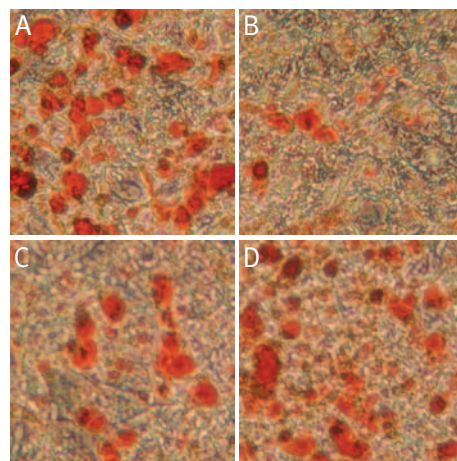
Genistein (#G-300) inhibits the Tyrosine phosphorylation of 28 kDa protein. HeLa cells were grown to 70% confluency, and the cells serum starved for 18 h. The cells were preincubated for 30 min in presence or absence of 500 nM Genistein and stimulated for 2 h with or without 20% fetal calf serum. The cell extracts were analyzed by Western blotting by probing with phospho-Tyr specific antibodies.

SB 203580: MAPK Homolog - p38 Inhibitor

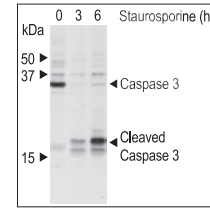


SB203580 (#S-370) inhibits the phosphorylation of Elk1 and Atf2 caused by p38 MAPK. 3T3-L1 cells were grown to 70% confluency and then serum starved for 3 h. The cells were incubated for 2 h with various concentrations of SB203580 and stimulated with 5 μ M **Anisomycin** (#A-520). The cell proteins were resolved by SDS PAGE, probed with anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-Elk1 and anti-phospho-Atf2. The phosphorylation of Elk1 and Atf2 was reduced as a function of SB203580 concentration.

Cyclosporin A: A Protein Phosphatase 2B Inhibitor and Immunosuppressant

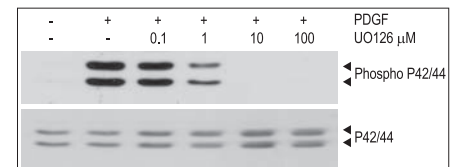


Staurosporine: A Protein Kinase Inhibitor and Apoptosis Inducer



Staurosporine (#S-350) induces apoptosis and inhibits phosphorylation. Jurkat cells were grown to 70% confluency, then 2 μ M Staurosporine or vehicle was added for 3 or 6 hours. At the end of the incubation period, the cells extracts were probed for Caspase 3 and cleaved Caspase 3 with specific antibodies.

U0126: A Specific MAPK Pathway Inhibitor



U0126 (#U-400) inhibits P42/44 MAPK phosphorylation. C6 glioma cells were grown to 70% confluence and then serum starved for 1.5 h. The cells were then incubated for 2 h with various concentrations of U0126 as indicated in the picture and stimulated with or without 7ng/ml PDGF-AA. The cell proteins were resolved by SDS PAGE, probed with anti phospho-P42/44 MAPK (upper panel) or with anti P42/44 MAPK (lower panel). The inhibition of P42/44 MAPK phosphorylation by U0126 increased respectively with the increase in U0126 concentration.

Cyclosporin A (#C-900) inhibits the antiadipogenic effect of Ca²⁺ dependent protein phosphatase (calcineurin). 3T3-L1 preadipocytes were induced to undergo adipocyte differentiation with 1 μ g/ml insulin for 4 days (A, B and C). Cells were additionally treated for the first 4 days of differentiation with 1 μ M **Ionomycin** (#I-700) (B-D) in the presence of 10 ng/ml (C) or 50ng/ml (D) Cyclosporin A. After 10 days the cells were stained with Oil Red O and visualized with light microscopy.

signaling cascades activated by TCR-dependent T cell activation.^{27, 28}

Ca²⁺-dependent Signaling and the Elusive CRAC Channel

A sustained elevation of intracellular Ca²⁺ is an indispensable step for a productive T cell activation. Elevated intracellular Ca²⁺ levels need to be maintained for several hours in order to permit downstream signaling events such as NF-AT translocation to the nucleus.¹⁰ This sustained Ca²⁺ elevation is accomplished by the concerted action of several receptors, kinases and ion channels.

As mentioned above, PLC γ 1 catalyzes the formation of IP₃ which binds to the IP₃ receptor (IP₃R) located in the endoplasmic reticulum (ER) membrane. Activation of the IP₃R induces a brief spike in intracellular Ca²⁺ concentration due to the release of Ca²⁺ from the ER stores. The continuous activation of the IP₃R depletes the ER Ca²⁺ stores, an event that triggers the opening of the Ca²⁺ release-activated-Ca²⁺ channel (CRAC) in the plasma membrane by an as yet unknown mechanism. Only the opening of the plasma membrane CRAC channel permits entry of sufficient Ca²⁺ in order to maintain the required high intracellular levels. Because Ca²⁺ entry depolarizes the cell and thus reduces the driving force for further Ca²⁺ entry, a sustained Ca²⁺ entry can only be achieved by the participation of a voltage-gated K⁺ channel. In the case of T cells, this channel is the voltage-dependent K⁺ channel K_v1.3. The K_v1.3 channel is located in the plasma membrane and opens in response to the membrane depolarization caused by Ca²⁺ entry. Opening of the channel produces an efflux of K⁺ thus restoring the negative membrane potential that will permit further Ca²⁺ entry.¹¹ Another channel involved in the regulation of Ca²⁺ entry is the intermediate conductance Ca²⁺-activated K⁺ channel, K_{ca}3.1 (also known as SK4 or IK_{ca}1). This channel is structurally similar to the K_v1.3 channel but it opens in response to a rise in intracellular Ca²⁺ instead of membrane depolarization.

The pivotal role of the K⁺ channels in controlling T cell activation and proliferation was demonstrated by the use of several channel-blocking compounds such as **Charybdotoxin**, **Margatoxin** and **Stichodactyla toxin** (ShK) that were able to block T cell function both *in vitro* and *in vivo*.²²⁻²⁴ As K_v1.3 channel levels were shown to be upregulated in T cells infiltrating the brain of multiple sclerosis patients, treatment of these patients with K⁺-channel blockers could offer a very attractive therapy in the near future.²⁴

Remarkably, about 75% of the T cell activation-regulated genes show a dependence on Ca²⁺ influx through the CRAC channels. The importance of Ca²⁺ entry through CRAC channels

in T cell activation is highlighted by the fact that patients with a complete absence of CRAC channel activity show a phenotype of severe immunodeficiency.^{13,14}

Although a great deal is known regarding the biophysical, electrophysiological and pharmacological characteristics of the CRAC channel, the molecular identity of the channel remains elusive. Several members of the transient receptor potential (TRP) channel superfamily have been proposed to be good candidates to encode the CRAC channel including TRPC1,¹⁵ TRPC3,¹⁶ TRPV6,¹⁷ and TRPM2,¹⁸ as well as members of the voltage-gated Ca²⁺ (Ca_v) channel family.¹⁹⁻²¹

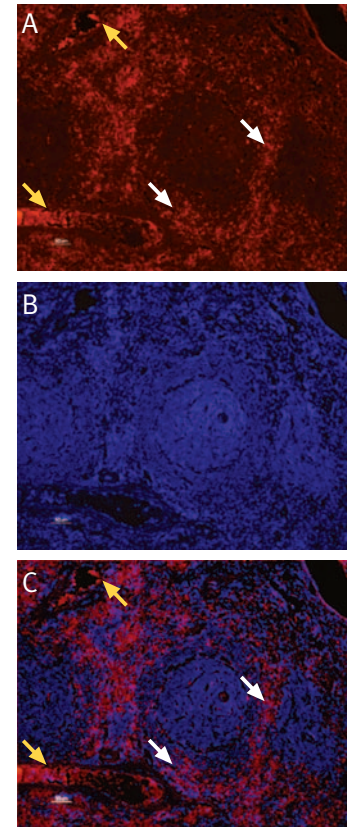
The rise in intracellular Ca²⁺ permits the activation of the phosphatase calcineurin, among others. Calcineurin dephosphorylates NF-AT in the cytosol inducing its activation and allowing it to translocate to the nucleus.¹² The importance of the calcineurin-NF-AT pathway is underscored by the fact that the widely used immunosuppressants **Cyclosporin A** and **FK-506** target this particular interaction. Their immunosuppressive activity stems from the fact that inhibition of NF-AT activation and translocation to the nucleus results in the inhibition of the transcription of key genes involved in the immune response.²⁵

Concluding Remarks

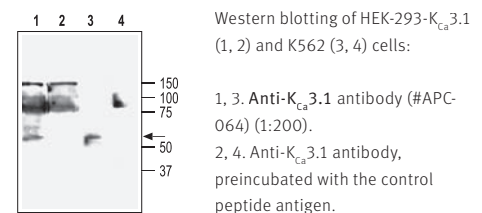
A great deal of progress has been made in the last few years towards elucidating the molecular mechanisms involved in the control of T cell activation. The major signaling cascades are now fairly well understood and some of the complex interactions between them have been also deciphered.

What is certain is that activating a T cell is not a simple matter, but given the potentially disastrous results of an inappropriate T cell response, this may be a good thing.

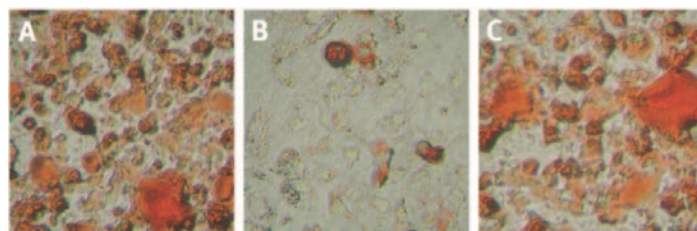
Expression of K_{ca}3.1 in Rat Spleen



Immunohistochemical staining of K_{ca}3.1 with Anti-K_{ca}3.1 antibody (#APC-064) in rat spleen. (A) and (C) Secondary (activated) follicle in the spleen white pulp showing intense staining of Marginal Zone and Periarteriolar T-lymphocytes (white and yellow arrows, respectively); note that cells in the red pulp and B lymphocytes in the germinal center are not stained. (B) The counterstain is Hoechst 33324.

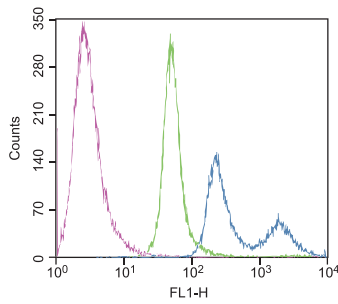


Protein Phosphatase 2B Inhibitor - Immunosuppressant



FK-506 (#F-900) inhibits the antiadipogenic activity of calcineurin. 3T3-L1 preadipocytes were induced to undergo adipocyte differentiation with 1 μg/ml insulin for 4 days (A, B and C). As indicated cells were additionally treated for the first 4 days of differentiation with 1 μM Ionomycin (#I-700) (B and C) in presence (C) or absence (A and B) of 5 ng/ml FK-506. After 10 days the cells stained with Oil Red O and visualized with light microscopy. A.

Expression of $K_v1.3$ in Normal and Activated T-cells

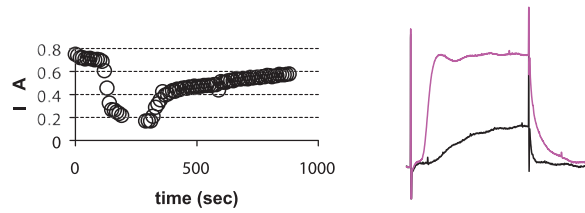


Human Jurkat cells (a T cell leukemia line) were either left untreated or treated with 1mg/ml phytohemagglutinin A (PHA) for 24 hours. Surface expression of the $K_v1.3$ channel was studied using an Anti- $K_v1.3$ -extracellular antibody directly labeled with the fluorophore FITC (#APC-101-F) and analyzed in a flow cytometer. Untreated and unstained cells are shown in violet. Untreated cells stained with the antibody are shown in green while PHA- treated stained cells are shown in blue. Note that PHA-treated cells show a marked surface upregulation of the $K_v1.3$ channel.

References:

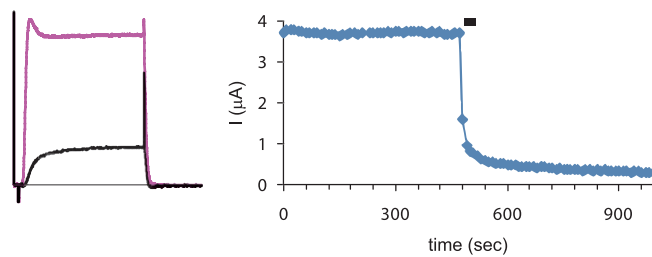
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rMargatoxin: A Potent Blocker of $K_v1.3$ Channels



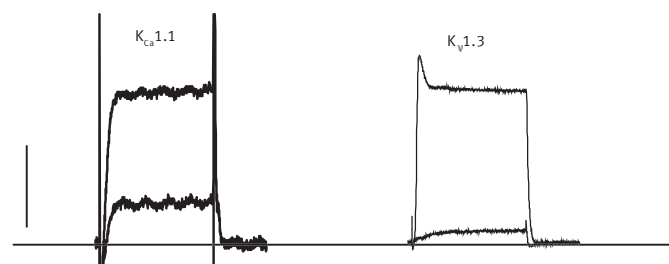
Inhibition of heterologously expressed $K_v1.3$ channels by 10 nM rMargatoxin (#RTM-325). Left: time course of current amplitude change, upon application of toxin. Currents were recorded with extracellular ND96 solution and were elicited every 10 seconds by 100 ms pulse to 0 mV (holding potential was -100 mV). The bar represents period of toxin perfusion. Right: superimposed traces before (violet) and during (black) application of 10 nM rMargatoxin.

Stichodactyla Toxin (Shk): A Voltage-Dependent K^+ Channel Blocker



Irreversible inhibition of $K_v1.3$ channel current expressed in *Xenopus* oocytes by 25 nM Stichodactyla Toxin (Shk) (#S-400). Using TEVC, membrane potential was held at -100 mV and currents were elicited by 100 ms voltage steps to 0 mV delivered every 10 seconds. Left: Traces of currents before (violet) and during bath perfusion of 25 nM Shk. Right: The time course of amplitude change in the same experiment. The period of toxin application is indicated by the horizontal bar.

rCharybdotoxin: A Potent Blocker of Some Ca^{2+} and Voltage-Dependent K^+ Channels



The effect of 100 nM rCharybdotoxin (#RTC-325) on $K_{Ca}1.1$ (left) and $K_v1.3$ (right, co-expressed with $K_v\beta1.1$) channels, expressed in *Xenopus* oocytes. In both cases the currents were recorded with standard ND96 solution (see above), Membrane holding potential was -100 mV, stepped every 15 s. to $+20$ mV for 100 ms. The vertical bar represents 0.1 and $3 \mu A$ for $K_{Ca}1.1$ and $K_v1.3$ respectively.

Related Products

Compound	Product #	Applications Tested
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Ion Channel Antibodies

Anti-K _v 1.3	APC-002	WB, IH
Anti-K _v 1.3 Extra	APC-101	WB, IC, IFC
Anti-K _v 1.3 Extra-FITC	APC-101-F	FC
Anti-K _{ca} 3.1	APC-064	WB, IH
Anti-IP3R1	ACC-019	WB, IC
Anti-TRPC1	ACC-010	WB, IH
Anti-TRPC3	ACC-016	WB, IH
Anti-TRPC4	ACC-018	WB, IH
Anti-TRPC5	ACC-020	WB
Anti-TRPC6	ACC-017	WB, IH
Anti-TRPV6	ACC-036	WB

Compound	Product #	Blocker of
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Ion Channel Blockers

rCharybdotoxin	RTC-325	K _{ca} 1.1 (BK _{ca}); K _{ca} 3.1 (SK4); K _v 1.3
rMargatoxin	RTM-325	K _v 1.3
Stichodactyla (ShK)	S-400	Several K _v Channels
rMaurotoxin	RTM-340	K _{ca} 3.1 (SK4); Kv1.2
rAgitoxin-1	RTA-150	K _v 1.3
rAgitoxin-2	RTA-420	K _v 1.1; K _v 1.3; K _v 1.6
rAgitoxin-3	RTA-390	K _v 1.1; K _v 1.3; K _v 1.6
rHongotoxin-1	RTH-400	K _v 1.1; K _v 1.2; K _v 1.3; K _v 1.6

Compound	Product #	Known Function
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Cell Signaling

Genistein	G-300	Tyrosine Kinase Inhibitor
Staurosporine	S-350	Protein Kinase Inhibitor (non specific)
K252a	K-150	Protein Kinase Inhibitor (non specific)
K252b	K-170	Protein Kinase Inhibitor (non specific)
Chelerythrine	C-400	PKC Inhibitor
SB203580	S-370	MAPK Inhibitor (p38 specific)
U0126	U-400	MAPK Inhibitor (ERK Inhibitor)
Cyclosporin A	C-900	Calcineurin Inhibitor
FK-506	F-900	Calcineurin Inhibitor
A23187	A-600	Calcium Ionophore
Ionomycin	I-700	Calcium Ionophore
Thapsigargin	T-650	Intracellular Calcium Mobilizer
Thapsigargin epoxide	T-670	Non active analog of Thapsigargin

Taipoxin Presynaptic Phospholipase A2 Neurotoxin

Taipoxin, is a lethal neurotoxin protein isolated from the venom of the Australian taipan snake (*Oxyuranus s. scutellatus*) (LD₅₀ is 2 µg/kg in mouse). Taipoxin is a non-covalent ternary glycoprotein which dissociates completely at low pH or high ionic strength. One of the subunits has Phospholipase A2 activity.^{1,2}

Intoxicated animals die of asphyxia by neuromuscular blockage of the respiratory muscles caused by a complete inhibition of neurotransmitter release. In the central nervous system, Taipoxin blocks the synaptic vesicles recycling by inhibiting the neuronal uptake pathway, interacting with neuronal Pentraxin and Pentraxin receptor during synapse formation and remodeling.^{1,3-5}

In neuronal cultures, Taipoxin facilitates Ca²⁺-dependent synaptic vesicle exocytosis and causes a complete depletion of stored neurotransmitters, resulting in synaptic transmission blockage. Taipoxin, in the nanomolar range, causes swelling of nerve terminals and redistributing synaptic vesicle proteins (Synaptotagmin 1). Recently, it was proposed that the mechanism of action involves the toxin's phospholipase activity which hydrolyzes the membrane phospholipids to lysophospholipids and fatty acids. The existence of these products in the membrane promote the vesicle-membrane fusion leading to initial massive exocytosis.^{7,8} This activity makes Taipoxin a powerful tool for the study of exocytosis.

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Taipoxin (#T-460) changed the actin filaments distribution of the growing neurite tip bulge in PC12 cells. The cells were challenged with 50 ng/ml mNGF 2.5S (#N-240) for 3 days followed by stimulation with (B) 10nM, (C) 20nM or without (A) Taipoxin for 1h. Then the actin filaments were labeled with rhodamine conjugated phalloidin and visualized using fluorescent microscopy.

