Involvement of protein kinases in the upregulation of acetylcholine release at endplates of α-bungarotoxin-treated rats

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1. ACh release from motor nerve endings in diaphragms of rats treated chronically with α-bungarotoxin (α-BuTX) is upregulated at the level of the individual endplate. Involvement of protein kinases in this mechanism of synaptic adaptation was investigated.

2. Miniature endplate potentials (MEPPs) and endplate potentials (EPPs) were recorded after μ-conotoxin treatment, which prevents muscle action potentials. The quantal content at endplates was calculated 'directly', i.e. by dividing the EPP amplitude by the MEPP amplitude.

3. Incubation of muscles from control and α-BuTX-treated rats with H-7, a protein kinase C (PKC) inhibitor, reduced MEPP amplitudes but had no clear effect on quantal contents. Polymyxin B, another PKC inhibitor, had a similar effect on muscles from α-BuTX-treated rats.

4. Incubation of muscles from α-BuTX-treated rats with K252a, a broad-spectrum protein kinase inhibitor of, amongst others, PKC, Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII) and neurotrophin receptor tyrosine kinases, resulted in a 30% decrease of the quantal content. However, K252a did not change the quantal content of controls. Incubations with the closely related compound K252b, which has an exclusively extracellular action, had a similar effect.

5. KN62, a specific inhibitor of CaMKII, decreased the mean quantal content of muscles from α-BuTX-treated rats by 18%.

6. Tyrphostin 51, a selective tyrosine kinase inhibitor, had no effect on quantal contents of muscles from α-BuTX-treated and control rats. However, it increased the frequency and amplitude of MEPPs in muscles from α-BuTX-treated rats, leaving those of controls unchanged.

7. The extent of reduction of quantal content, caused by K252a, K252b and KN62, varied between endplates of individual muscles from α-BuTX-treated rats; quantal contents at endplates with small MEPPs were more sensitive than those at endplates with large MEPPs.

8. It is concluded that PKC does not play a role in the mechanism of upregulation of ACh release at endplates of α-BuTX-treated rats. Instead, CaMKII and some tyrosine kinases in the presynaptic membrane, as well as in the cytoplasm, might be involved.

Chronic treatment of rats with α-bungarotoxin (α-BuTX), a blocker of ACh receptors (AChRs) in muscles, results in myasthenia gravis-like (MG) symptoms of muscle weakness. This condition of 'toxin-induced myasthenia gravis' (TIMG) leads to upregulation of ACh release from motor nerve terminals to an extent which at each individual endplate is correlated with the extent of the α-BuTX-induced loss of postsynaptic sensitivity for ACh (Plomp, van Kempen & Molenaar, 1992). Upregulation of ACh release at the level of the individual endplate has also been demonstrated in muscle biopsies from MG patients and in muscles from rats with experimental auto-immune MG (Plomp, van Kempen,

De Baets, Graus, Kuks & Molenaar, 1995). We have suggested that the mechanism underlying upregulation of ACh release involves retrograde signalling from the muscle fibre to the nerve terminal (Plomp *et al.* 1992). Recent results indicate that the modulatory action of such a retrograde signal on the presynapse is brought about by the stimulation of a $[Ca^{2+}]_i$-dependent component of the ACh-release mechanism (Plomp, van Kempen & Molenaar, 1994).

Protein kinases are relevant candidates for such $[Ca^{2+}]_i$-sensitive components. At neuromuscular junctions of frogs, mice and rats, the stimulation of protein kinase C (PKC) by phorbol esters results in an increase of either spontaneous or evoked ACh release (for references see Van der Kloot & Molgó, 1994). Synaptic vesicle phosphoproteins could be the final targets of messengers that modulate neurotransmitter release, since evidence has accumulated that these proteins play a role in processes of trafficking, docking and membrane fusion of synaptic vesicles (Greengard, Valtorta, Czernik & Benfenati, 1993). For instance, from recent studies in the squid giant synapse it emerges that synapsin I, one of the vesicle phosphoproteins, binds synaptic vesicles to the cytoskeleton of the nerve terminal, thereby creating a store of neurotransmitter which is not directly available for release. When synapsin I is phosphorylated by Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII), unbinding of synaptic vesicles from the cytoskeleton results, which leads to an increase of neurotransmitter release (Llinás, Gruner, Sugimori, McGuinness & Greengard, 1991; Walaas & Greengard, 1991; Benfenati & Valtorta, 1993). Thus, stimulation of PKC or CaMKII, possibly via intracellular messengers in the nerve terminal, could be involved in the mechanism for upregulation of ACh release at myasthenic endplates.

Other protein kinases that could be involved in upregulation of ACh release at myasthenic endplates are the tyrosine kinases that are high-affinity receptors for neurotrophins, since it has been shown that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), besides their long-term trophic influence on cholinergic neurons (Oppenheim, Qin-Wei, Prevette & Yan, 1992; Wong, Arriaga, Ip & Lindsay, 1993), have an acute increasing effect on ACh release at developing *Xenopus* neuromuscular junctions *in vitro* (Lohof, Ip & Poo, 1993). If receptor tyrosine kinases are involved in the upregulation of ACh release, this would suggest a role for a neurotrophin as a retrograde messenger at myasthenic endplates. Furthermore, non-receptor tyrosine kinases could be involved since it has been shown that the activity of such kinases plays a role in synaptic plasticity (O'Dell, Kandel & Grant, 1991).

In order to assess a possible role of PKC, CaMKII or tyrosine kinases in the mechanism for upregulation of ACh release at TIMG endplates, we studied the *in vitro* effects of inhibitors of these enzymes. Part of this study has been published in abstract form (Plomp & Molenaar, 1994).

METHODS

Animal model for MG

Female Wistar rats, weighing about 100 g at the beginning of the experiment, were used. A dose of 3–5 μg α-BuTX (Biotoxins Inc., St Cloud, FL, USA), dissolved in physiological saline, was subcutaneously injected every 48 h for 3–4 weeks (Plomp *et al.* 1994). After the injection, symptoms of facial muscle weakness appeared within 1–2 h. Breathing was not markedly impaired. The symptoms slowly subsided in the next 8–10 h, probably due to insertion of newly synthesized AChRs. On the next, 'toxin-free', day the rats showed no symptoms of muscle weakness and were able to eat and drink so that growth during the treatment was only moderately impaired. When the symptoms of weakness of individual rats, assessed by a score system at 3 h after each α-BuTX injection, were too mild or too severe, the dose of the following injection was increased or decreased by 0·5 μg. Control rats received physiological saline. The TIMG procedure had been approved by the Animal Ethical Committee at Leiden (Approval number 10614/17) as required by the Dutch Law on Animal Experiments. The rats were killed by an overdose of ether and the diaphragms, with their nerves, were dissected rapidly. The left hemidiaphragms were used in the experiments.

Protein kinase inhibitors

The protein kinase inhibitors used are listed in Table 1. Two (non-selective) inhibitors of PKC were used: (1) 120 μM H-7, an isoquinoline derivate (K_i, 6 μM: Hidaka & Kobayashi, 1992; K_i for cAMP- and cGMP-dependent protein kinases of 3·0 μM and 5·8 μM, respectively: Hidaka & Kobayashi, 1992); and (2) 7–30 μM polymyxin B (PMB), an antibiotic (K_i, 1·8 μM: Mazzei, Katoh & Kuo, 1982).

Two CaMKII-inhibiting compounds were tested: (1) 200 nM K252a, a broad-spectrum kinase inhibitor from microbial origin, which also inhibits cAMP-dependent protein kinase, cGMP-dependent protein kinase and myosin light chain kinase, as well as PKC ($K_i < 25$ nM: Hashimoto *et al.* 1991; Hidaka & Kobayashi, 1992); and (2) 10 μM KN62, which specifically inhibits CaMKII (K_i of about 1 μM: Hidaka & Kobayashi, 1992).

At nanomolar concentrations, K252a also inhibits the receptor tyrosine kinases for neurotrophic factors such as NT-3 and BDNF (Knüsel & Hefti, 1992; Tapley, Lamballe & Barbacid, 1992). The use of the closely related compound K252b allowed discrimination between extra- and intracellular effects of K252 compounds because it does not freely pass the cell membrane like K252a (Nagashima, Nakanishi & Matsuda, 1991). At concentrations higher than 100 nM, K252b has been shown to inhibit the tyrosine kinase receptors as well as the long-term trophic effects of NT-3 and BDNF on neurons, while at low concentrations (1–100 nM) it potentiates the trophic effects of NT-3 but not BDNF, presumably via a stimulatory effect on the receptor tyrosine kinases (Knüsel & Hefti, 1992; Cheng, Barger & Mattson, 1994).

Tyrphostin compounds are selective inhibitors of protein tyrosine kinases with different inhibiting potencies for the individual tyrosine kinases (Levitzki & Gazit, 1995). Although little is known about the possible inhibiting properties of tyrphostins acting on

Table 1. Protein kinase inhibitors used in the present study

Substance	Supplier	Concentration in bath (μM)	Concentration in stock (mM)	Solvent
PMB (polymyxin B sulphate)	Sigma	7–30	10	H_2O
H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine)	Sigma	120	60	H_2O
KN62 (1-[*N*-*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine)	Euro Biochem, Bierges, Belgium	10	10	DMSO
K252a (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[a, g]-cycloocta[cde]trinden-1-one)	Euro Biochem	0·2	0·2	DMSO
K252b (8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-carboxylic acid-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[a, g]-cycloocta[cde]trinden-1-one)	Euro Biochem	0·2	0·2	DMSO
Tyrphostin 51 (2-amino-1,1,3-tricyano-4-[3′,4′,5′-tri-hydroxyphenyl]-butadiene)	Sigma	50	50	DMSO

the neurotrophin receptor tyrosine kinase, we tested the effect of 50 μM tyrphostin 51 (also named AG183), a potent epidermal growth factor receptor inhibitor (K_i, 0·8 μM: Gazit, Yaish, Gilon & Levitzki, 1989).

Bathing solutions, muscle action potential elimination and experimental protocol

After dissection, the muscles were kept at room temperature (20–22 °C) in 95% O_2–5% CO_2-saturated Ringer solution containing (mM): NaCl, 116; KCl, 4·5; $NaHCO_3$, 23; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; and glucose, 11.

μ-Conotoxin, a component of the venom from the marine snail *Conus geographus*, abolishes muscle action potentials by blocking voltage-gated Na^+ channels of muscle but not those of nerve and thus uninfluenced endplate potentials (EPPs) can be recorded at normal resting membrane potentials (Hong & Chang, 1989; Di Gregorio, Fesce, Cereser, Favaro & Fiori, 1989). Measurements in cut-fibre preparations have shown that μ-conotoxin itself has no effect on the quantal content (Hong & Chang, 1989; Plomp *et al.* 1992).

Hemidiaphragms were pinned out in a preparation dish and were incubated for 40 min in 5 ml of Ringer solution containing 2·3 μM μ-conotoxin (synthetic, type GIIIB; Scientific Marketing Associates, Barnet, Herts, UK), while 95% O_2–5% CO_2 was blown over the surface of the bathing medium. Subsequently, the muscles were superfused with Ringer solution, without μ-conotoxin, circulating via a roller pump from a stock of about 100 ml which was bubbled with 95% O_2–5% CO_2. The temperature of the Ringer solution in the preparation dish was kept at 26–28 °C. Electrophysiological measurements were performed as described below. During the actual recordings the circulation of Ringer solution was interrupted. This first set of measurements was completed within 1·5 h. Thereafter, the muscle was incubated for 1 h in 5 ml Ringer solution containing one of the protein kinase inhibitors from Table 1 and 2·3 μM μ-conotoxin in order to maintain the blockade of muscle action potentials. Subsequently, the medium was replaced by 5 ml kinase inhibitor solution without μ-conotoxin, and a second series of electrophysiological measurements were carried out during the next 1–2 h. When DMSO was used as the protein kinase inhibitor solvent the stock solution was diluted to give a final bath concentration of 0·1%. In that case, the first μ-conotoxin incubation and the first set of measurements were done in Ringer solution containing 0·1% DMSO.

Electrophysiological recordings

Our methods of microelectrode measurements, signal analysis and calculation of quantal content have been described previously (Plomp *et al.* 1992, 1994). In short, muscle fibres were impaled near the endplate with a glass capillary microelectrode and miniature endplate potentials (MEPPs) and EPPs (at 0·3 Hz) were recorded at the same endplate. Off-line analysis was done on computer to determine amplitudes of the EPPs and MEPPs. From this the quantal content was calculated after normalization of the amplitudes to −75 mV resting membrane potential and correction of the EPP amplitude for non-linear summation (McLachlan & Martin, 1981).

Statistics

The statistical significance of differences between mean values was tested with Student's *t* test, paired or unpaired, where appropriate.

RESULTS

The grand mean values of the electrophysiological parameters, measured in muscles from the TIMG rats and their controls, are given in Table 2. The picture that emerged was similar to that described in previous papers (Plomp *et al.* 1992, 1994) showing the following typical TIMG characteristics: (1) reduction of the MEPP amplitude; (2) upregulated quantal content; (3) decrease of the MEPP frequency; and (4) a small decrease of the EPP amplitude. Furthermore, the inverse correlation between quantal content and MEPP amplitude of individual TIMG endplates that has been described previously (Plomp *et al.* 1992, 1994) was confirmed in the present experiments (Fig. 1).

Table 2. Effect of protein kinase inhibitors on electrophysiological parameters in rat diaphragm

	TIMG			Control			
			H-7				
Parameters	a 0 μM	b 120 μM	b/a	c 0 μM	d 120 μM	d/c	a/c
MEPP amplitude (mV)	0·28 ± 0·02	0·19 ± 0·01	0·69*	0·48 ± 0·04	0·31 ± 0·04	0·66**	0·58††
MEPP frequency (s^{-1})	1·08 ± 0·07	0·91 ± 0·11	0·84	1·50 ± 0·09	1·36 ± 0·07	0·90	0·72†
EPP amplitude (mV)	23·39 ± 0·75	18·18 ± 0·28	0·78**	28·07 ± 1·03	23·74 ± 1·72	0·85*	0·83†
Quantal content	130 ± 5·5	127 ± 5·4	0·98	94 ± 8·2	109 ± 4·4	1·16*	1·38†
			K252a				
	a 0 nM	b 200 nM	b/a	c 0 nM	d 200 nM	d/c	a/c
MEPP amplitude (mV)	0·31 ± 0·03	0·26 ± 0·01	0·85	0·57 ± 0·03	0·55 ± 0·02	0·96	0·54††
MEPP frequency (s^{-1})	1·33 ± 0·08	1·2 ± 0·14	0·90	1·52 ± 0·28	1·47 ± 0·20	0·96	0·87
EPP amplitude (mV)	22·00 ± 0·47	16·50 ± 0·72	0·75**	28·68 ± 0·69	26·70 ± 0·51	0·93	0·77†
Quantal content	111 ± 6·1	78 ± 4·1	0·70*	79 ± 2·3	80 ± 4·1	1·01	1·40††
			K252b§				
	a 0 nM	b 200 nM	b/a	c 0 nM	d 200 nM	d/c	a/c
MEPP amplitude (mV)	0·29 ± 0·02	0·31 ± 0·03	1·05	0·57 ± 0·04	0·61 ± 0·05	1·07	0·51††
MEPP frequency (s^{-1})	1·07 ± 0·12	1·08 ± 0·09	1·00	1·92 ± 0·17	1·61 ± 0·10	0·84	0·56††
EPP amplitude (mV)	23·63 ± 0·88	20·01 ± 0·90	0·85	28·56 ± 1·14	28·35 ± 1·27	0·99	0·83††
Quantal content	121 ± 6·1	90 ± 3·1	0·75**	79 ± 2·3	73 ± 3·7	0·93	
		KN62					
	a 0 μM	b 10 μM‡	b/a				
MEPP amplitude (mV)	0·25 ± 0·02	0·26 ± 0·02	1·02				
MEPP frequency (s^{-1})	1·36 ± 0·23	1·84 ± 0·39	1·36				
EPP amplitude (mV)	22·59 ± 0·53	19·21 ± 0·79	0·85*				
Quantal content	133 ± 5·2	109 ± 6·7	0·82*				
			Tyrphostin 51‖				
	a 0 μM	b 50 μM	b/a	c 0 μM	d 50 μM	d/c	a/c
MEPP amplitude (mV)	0·32 ± 0·03	0·49 ± 0·04	1·55*	0·61 ± 0·02	0·65 ± 0·05	1·07	0·53††
MEPP frequency (s^{-1})	0·99 ± 0·06	1·85 ± 0·23	1·86*	1·69 ± 0·19	1·70 ± 0·10	1·00	0·59††
EPP amplitude (mV)	22·94 ± 1·29	30·27 ± 1·25	1·32**	28·32 ± 0·28	28·45 ± 1·07	1·00	0·81††
Quantal content	108 ± 2·4	104 ± 5·9	0·96	75 ± 2·8	71 ± 3·8	0·95	1·45††

Values are grand means ± S.E.M. of the muscle means; columns a and c represent values before, and b and d represent values after incubation with the test compound. Each experimental group consisted of four muscles, ten to fifteen endplates were sampled per muscle. EPPs used for the calculation of the quantal content were recorded at 0·3 Hz nerve stimulation. The mean amplitude of MEPPs and EPPs of the individual endplates was normalized to the standard resting membrane potential of −75 mV before the calculation of the muscle mean; EPP amplitudes were not yet corrected for non-linear summation. ‡ One of the muscles was incubated with 20 μM KN62. § Tested on six TIMG and six control muscles. ‖ Tested on five TIMG and five control muscles. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; Student's paired t test on pairs of muscle means before and after the incubation with the test compound. †$P < 0{\cdot}05$, ††$P < 0{\cdot}01$; Student's unpaired t test on grand means.

The results of the incubations with protein kinase inhibitors are given below and are summarized in Table 2.

H-7

Incubation of TIMG and control muscles with 120 μM H-7, a PKC inhibitor, resulted in a significant reduction of the mean MEPP ($P < 0.05$) and EPP ($P < 0.01$) amplitudes by 15–30%. Although H-7 did not change the mean quantal content of the TIMG muscles, it slightly increased the mean quantal content of the control group.

In order to be able to consider the effect of H-7 on the inverse relationship between quantal content and MEPP amplitude, endplates were classified with regard to the mean of their MEPP amplitudes in classes with a width of 0·1 mV, and the mean quantal content of the endplates within the classes was calculated. From these data, curves demonstrating the relationship between quantal content and MEPP amplitude were plotted. In Fig. 1*A* and *B* it is shown that the quantal contents in the endplate classes from TIMG as well as control muscles were not significantly influenced by 120 μM H-7. Apparently, the just significant increase ($P = 0.04$) of the mean value of the quantal content of the control group was due to the addition of slight, not significant, changes in the individual endplates classes. The reduction in mean MEPP amplitude by H-7 in TIMG as well as in control muscles is reflected in the curves by the loss of points in the higher MEPP amplitude range, i.e. at the right-hand side of the TIMG and control curves. An inverse relationship, although less steep, between quantal content and MEPP amplitude of endplates was also found within the control muscles as previously reported and discussed (Plomp *et al.* 1992).

Polymyxin B

PMB, another PKC inhibitor, was used at 7, 15 and 30 μM; each concentration was tested on a different TIMG diaphragm. The mean values of the MEPP amplitude and the quantal content of these three muscles before the incubation with PMB were 0.32 ± 0.03 mV and 137 ± 7.8 (15 endplates per muscle), respectively. Like H-7, PMB decreased MEPP and EPP amplitudes. Incubation of the three TIMG muscles with PMB (5–15 endplates per muscle) resulted in a concentration-dependent decrease of the mean amplitude of the MEPPs to 50% at 30 μM, compared with the value before the incubation (Fig. 2). In a number of endplates of the TIMG muscle incubated with 30 μM PMB, MEPPs could not be detected while the characteristics of the EPPs confirmed correct placement of the micro-electrode. Data from these endplates were discarded. PMB increased the frequency of the MEPPs by 279, 186 and 33% at 7, 15 and 30 μM, respectively (not illustrated in Fig. 2). The mean quantal contents of the TIMG muscles were not influenced by PMB (Fig. 2).

K252a

TIMG and control hemidiaphragms were incubated with a 200 nM solution of the broad-spectrum kinase inhibitor K252a (Table 2). In the TIMG group, K252a induced significant decreases of 25% ($P < 0.01$) and 30% ($P < 0.05$) of the mean values of the EPP amplitude and the quantal content, respectively. The mean values of the MEPP amplitude and frequency were unchanged. None of the electrophysiological parameters measured in the control muscles was changed significantly by K252a.

Figure 1*C* shows that the inverse relationship between quantal content and MEPP amplitude in TIMG muscles was disturbed by K252a. After K252a incubation, all the endplates, irrespective of the class of MEPP amplitude, had quantal contents in the range of the mean control value, i.e. about 80. Thus, the quantal contents at TIMG endplates with small MEPPs were reduced to a larger extent by K252a than those at TIMG endplates with large MEPPs. K252a exerted no effect on the inverse relationship between quantal content and the amplitude of the MEPP in the control group (Fig. 1*D*).

K252b

The effect of incubation of muscles with a 200 nM solution of the K252b analogue was similar to that of K252a. It also reduced the mean value of the quantal content of TIMG muscles significantly by 25% ($P < 0.01$), while it did not alter any of the measured electrophysiological parameters of control muscles (Table 2). Similarly, K252b disturbed the inverse relationship between the quantal content and the MEPP amplitude at TIMG endplates (Fig. 1*E*), while it had no influence on the curve of quantal content *versus* MEPP amplitudes of endplate classes of control muscle (Fig. 1*F*). The quantal content of the classes of TIMG endplates with MEPPs between 0·1 and 0·3 mV was significantly ($P < 0.01$) reduced by K252b.

KN62

TIMG muscles were incubated with 10 μM KN62, a specific CaMKII inhibitor. This resulted in significant changes of some of the measured electrophysiological parameters (Table 2). Thus, the mean values of the EPP amplitude and quantal content were decreased by 15% ($P < 0.05$) and 18% ($P < 0.05$), respectively.

The effect of KN62 on the inverse relationship between quantal content and MEPP amplitude in the TIMG muscles is shown in Fig. 1*G*. The quantal content in the classes of endplates with the smallest MEPPs, i.e. between 0·1 and 0·3 mV, was significantly ($P < 0.01$) reduced by KN62. One of the TIMG muscles was incubated with 20 μM KN62. The magnitude of the effect of KN62 at this concentration on the mean quantal content of the TIMG muscle was not different from that at 10 μM KN62. Therefore, the parameters of this muscle were included into the grand means.

Tyrphostin 51

Tyrphostin 51 (50 μM) was tested on five TIMG and five control muscles. Surprisingly, it induced a significant 55%

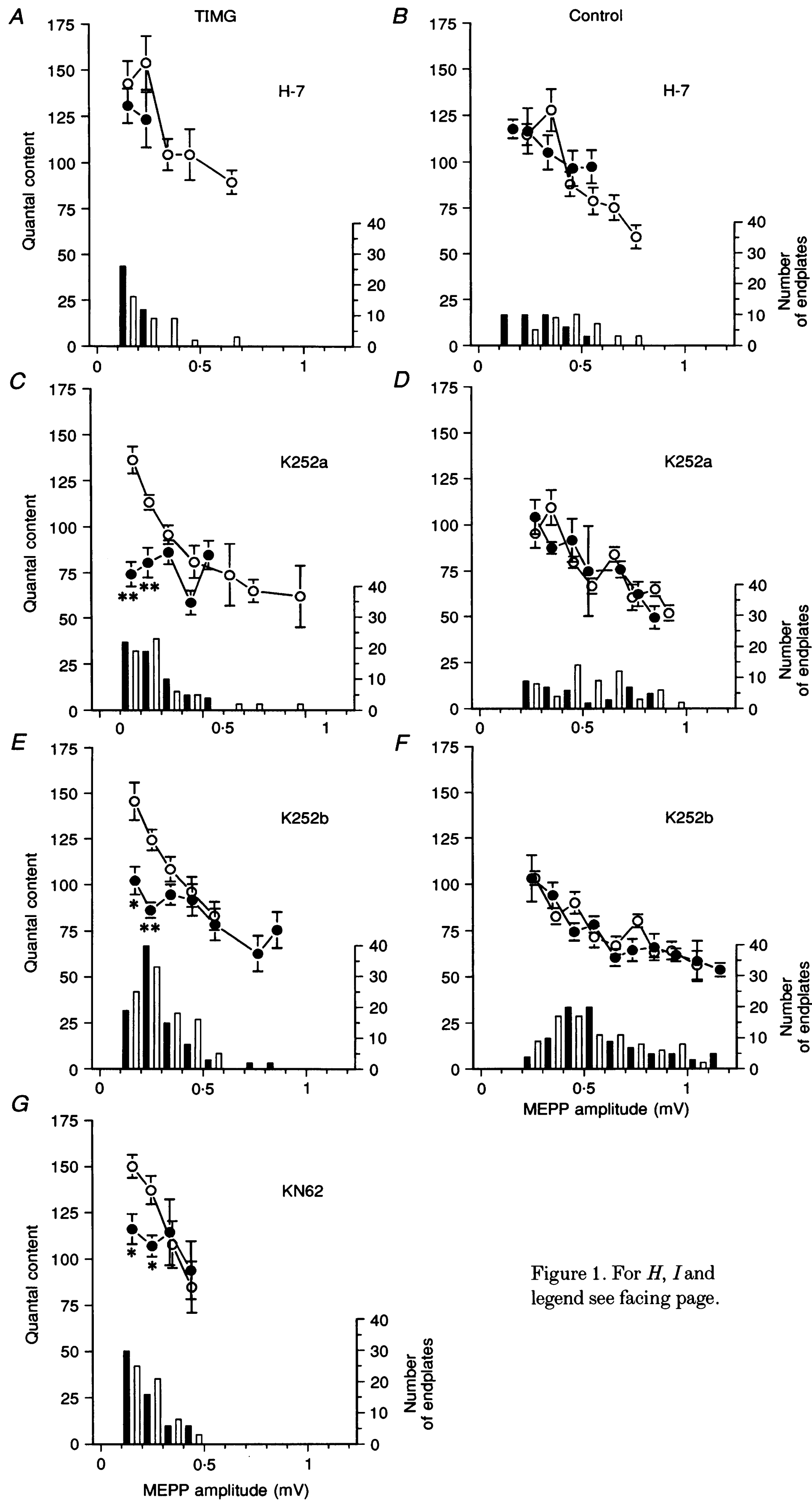

Figure 1. For *H*, *I* and legend see facing page.

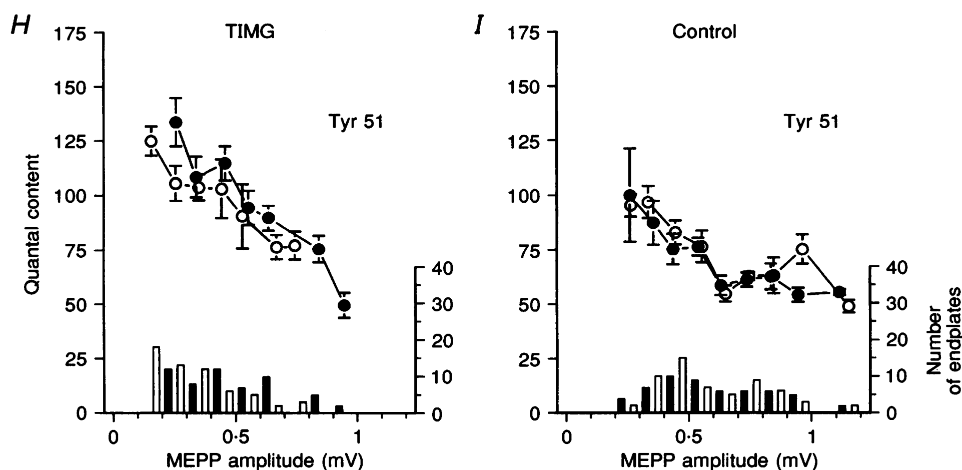

Figure 1. Effect of protein kinase inhibitors on the inverse relationship between quantal content and MEPP amplitude

Correlation between quantal content and MEPP amplitude in TIMG (*A*, *C*, *E*, *G* and *H*) and control (*B*, *D*, *F* and *I*) rat hemidiaphragms. Each experimental group consisted of four rats (except the K252b and tyrphostin 51 groups which consisted of six and five rats, respectively); ten to fifteen endplates were sampled per muscle. Endplates were classified with regard to their MEPP amplitudes in classes with a width of 0·1 mV. The mean quantal content within these classes was plotted against the mean MEPP amplitude within a class. The data points depict the quantal content *vs.* MEPP amplitude correlation before (○) and after (●) incubation with 120 μM H-7 (*A* and *B*), 200 nM K252a (*C* and *D*), 200 nM K252b (*E* and *F*), 10 μM KN62 (*G*) or 50 μM tyrphostin 51 (Tyr 51; *H* and *I*). The error bars of the symbols represent the S.E.M. within a class. The number of endplates of each class is indicated by the open (before incubation) and filled (after incubation) bars below the curves. Student's *t* test for significant difference from the mean value before protein kinase inhibition: $*P < 0{\cdot}01$, $**P < 0{\cdot}001$.

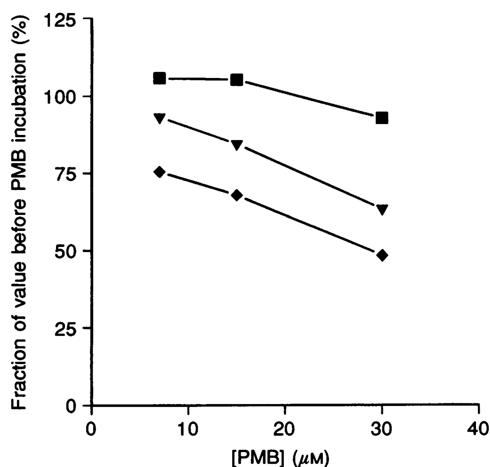

Figure 2. Effects of PMB on electrophysiological endplate parameters of TIMG hemidiaphragms

Effect of 7, 15 and 30 μM PMB on the mean quantal content (■), EPP amplitude (▼), and MEPP amplitude (◆) of TIMG hemidiaphragms. Each PMB concentration was tested on one TIMG muscle; data were sampled, before and after the PMB incubation, from five to fifteen endplates of each muscle. Data points represent the mean muscle values after PMB incubation as a percentage of the mean values before the incubation.

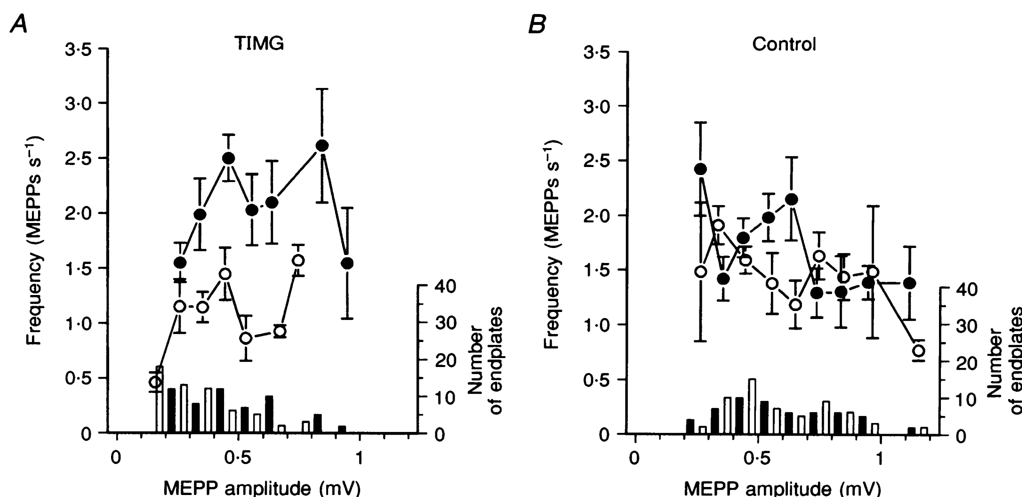

Figure 3. Effect of tyrphostin 51 on MEPP frequency

Effect of 50 μM tyrphostin 51 on the MEPP frequency of TIMG (*A*) and control (*B*) endplates. Endplates were classified with regard to their MEPP amplitudes in classes with a width of 0·1 mV. The mean MEPP frequency within these classes was plotted against the mean MEPP amplitude within a class. The data points depict the MEPP frequency *vs.* MEPP amplitude correlation before (○) and after (●) incubation with tyrphostin 51. Error bars represent the S.E.M. of the mean MEPP frequency in a class. The number of endplates in each class is indicated by the open (before incubation) and filled (after incubation) bars below the curves.

($P < 0.05$) increase of the mean MEPP amplitude of the TIMG muscles while leaving that of controls unchanged (Table 2). This observation suggests a postsynaptic effect of tyrphostin 51, selective for TIMG muscles. The mean amplitude of the EPP was increased to a more or less similar extent in TIMG muscles and remained unchanged in controls. Consequently, the mean quantal contents of TIMG and control muscles were unchanged. The correlation between quantal content and MEPP amplitude of endplates classes (Fig. 1*H* and *I*) also remained unchanged, although it appears that the TIMG curve shifted somewhat to the right (Fig. 1*H*) due to the potentiation of MEPP amplitudes. In spite of the lack of effect of tyrphostin 51 on the evoked quantal ACh release, it almost doubled the spontaneous quantal ACh release in TIMG muscles, while that in control muscles was not affected. The question arose as to whether this effect was due to the detection of MEPPs which had amplitudes below the detection threshold (about 0·1 mV) before the tyrphostin 51 incubation and which only became visible after the potentiation. Therefore, we plotted the mean MEPP frequency against the mean MEPP amplitude of endplates which were classified on behalf of their MEPP amplitude in classes with a width of 0·1 mV. Figure 3 shows that an increase in MEPP frequency of TIMG endplates occurred in all the endplate classes to a more or less similar extent, including those where the MEPPs were definitely detected before the incubation with tyrphostin 51 (i.e. the endplate classes in the tyrphostin curve with MEPP amplitudes above 0·3 mV).

DISCUSSION

In the present study, possible roles of PKC, CaMKII and tyrosine kinases in the mechanism of upregulation of ACh release at endplates of muscles from TIMG rats were considered. The major conclusion that can be drawn from the results of our experiments with several inhibitors of these protein kinases is that, although PKC is probably not involved, it is likely that CaMKII and a tyrosine kinase are involved in the ACh-release upregulating mechanism. In the discussion below, the effects of the various protein kinase inhibitors and their possible implications for the understanding of the phenomenon of upregulation of quantal content at single endplate level in TIMG will be dealt with.

Inhibition of PKC

The PKC inhibitor H-7 reduced the amplitudes of MEPPs at TIMG and control endplates, suggesting a postsynaptic effect of this compound as observed by others (Hirsh, Silinsky & Solsona, 1990). It is known that the nicotinic AChR has several phosphorylation sites; the postsynaptic effect of H-7 could thus be due to inhibition of the phosphorylation of these sites, thereby altering the characteristics of the receptor (Eusebi, Molinaro & Zani, 1985). Alternatively, changes in receptor characteristics could be the result of a direct effect of H-7 on the AChR (Reuhl, Amador, Moorman, Pinkham & Dani, 1992). It is possible that H-7 reduced the amplitudes of the smallest MEPPs in the TIMG endplates to values below the detection limit of our measuring set-up (about 0·1 mV),

thus causing loss of MEPPs. This could have resulted in overestimation of the mean MEPP amplitude in TIMG and thus in an underestimation of the mean quantal content. Loss of MEPPs on a large scale would be reflected in a clear decrease of the MEPP frequency in TIMG muscles. However, such a decrease was not observed after incubation of TIMG muscles with H-7.

The result that H-7 slightly increased the mean quantal content of control muscles conflicts, for unknown reasons, with the results of other studies. As mentioned in the introduction, stimulation of PKC by phorbol esters has been found to increase ACh release at the endplate, hence it was not expected that inhibition of PKC also would increase neurotransmitter release.

The concentration-dependent decrease in amplitude of MEPPs in TIMG endplates by the PKC inhibitor PMB confirmed the results of another study that reported a postsynaptic effect of PMB (Durant & Lambert, 1981). PMB also exerted a presynaptic effect, namely an increase of the MEPP frequency. The observation that the extent of this increase became less at the higher concentrations of PMB, strongly suggests that loss of MEPPs did occur, resulting in an overestimation of the mean values of the MEPP amplitudes and thus in an underestimation of the quantal content, as discussed above. In view of this, the true effect of PMB, at higher concentrations, could be a slight increase in mean quantal content in TIMG muscles instead of the lack of effect found in the present study. However, this bears no consequence for the conclusion that PKC is not involved in the mechanism for upregulation of quantal content at TIMG, because in that case it could be expected that the mean quantal contents would be dose-dependently reduced by PMB. Thus, although the lack of effect of PMB, at a concentration up to 30 μM, on the quantal content of TIMG endplates is not in agreement with the PMB-induced decrease in quantal content found by others at healthy frog endplates (Durant & Lambert, 1981), it suggests, together with the results obtained with H-7, that PKC activity is not involved in the mechanism of upregulation of ACh release at TIMG endplates. Furthermore, the negative result with H-7 seems to exclude involvement of at least two other kinases inhibited by the drug, namely cAMP- and cGMP-dependent protein kinase.

Inhibition of CaMKII

The observation that KN62 reduced the mean quantal content of TIMG muscles suggests that CaMKII is involved in the mechanism of ACh upregulation. This idea was further strengthened by the observation that KN62 had a differential effect on the TIMG endplates; while it reduced the quantal content at endplates that had the smallest MEPPs, and thus the largest extent of ACh release upregulation, it lacked any effect on the quantal content of TIMG endplates that had larger MEPPs. This disruption of the inverse relationship between quantal content and MEPP amplitude in TIMG muscles by KN62 suggests that the ACh release at endplates with the largest reduction in postsynaptic sensitivity for ACh was more dependent on CaMKII activity than ACh release at endplates with a higher postsynaptic sensitivity for ACh, i.e. in the range of that of healthy control endplates. As mentioned in the introduction, CaMKII can act as a modulator in the mechanism of neurotransmitter release by means of phosphorylation of synapsin I. In view of this and the present result obtained with KN62, we suggest that at TIMG endplates the activity of CaMKII is increased so that more synaptic vesicles are available for release. Furthermore, the present observation that another CaMKII inhibitor, K252a, had no effect on the quantal content of control muscles suggests that, under our experimental conditions, CaMKII activity is not required for 'normal' ACh release from motor nerve endings. In connection with this observation it is noteworthy that, at central synapses, synapsin I has been shown to be involved in circumstances of 'extra' neurotransmitter release but not in the 'normal' process (Rosahl *et al.* 1995).

With respect to a possible role for CaMKII in the mechanism of upregulation of ACh release at myasthenic endplates, it seems of interest that this protein kinase is also involved in the mechanism underlying long-term potentiation (LTP) at hippocampal synapses. Such a role for CaMKII was suggested by the observation that, in the hippocampus of mutant mice lacking CaMKII, LTP could not be produced (Silva, Stevens, Tonegawa & Wang, 1992). However, a recent study showed that CaMKII activity in the postsynaptic cell is underlying synaptic potentiation in hippocampal LTP (Pettit, Perlman & Malinow, 1994) and it was suggested that postsynaptic CaMKII activity could be involved in the release of a retrograde messenger which increases quantal content. As yet, our results do not enable us to conclude whether the increase of ACh release in TIMG is dependent on pre- or postsynaptic activity of CaMKII.

Inhibition of tyrosine kinases

The observation that K252a markedly reduced TIMG quantal contents, while leaving those of control muscles unchanged, strongly suggests that one or more protein kinases that are inhibited by K252a are involved in the mechanism of ACh upregulation. As with KN62, this was further strengthened by the way in which K252a disrupted the inverse relationship between quantal content and MEPP amplitude in TIMG, i.e. it particularly reduced the quantal contents at endplates that had the largest reduction in postsynaptic sensitivity for ACh and thus the largest extent of quantal content upregulation.

K252a is a broad-spectrum protein kinase inhibitor and thus several protein kinases could be involved in its reducing effect on upregulated quantal contents at TIMG endplates. From the present experiments with protein kinase inhibitors, it became clear that, although PKC was probably not involved, involvement of CaMKII seemed likely. However, the reducing effect of K252a on the grand

mean value of the quantal content of TIMG rats, as well as on the quantal content values of the endplate classes with the smallest MEPPs, was larger than that of KN62, suggesting that there is another protein kinase involved that acts synergistically with, or additionally to, CaMKII in the mechanism responsible for the upregulation of ACh release at TIMG endplates.

One way to discriminate between the protein kinases that are inhibited by K252a, was the use of K252b. This compound inhibits, with similar potency, the same protein kinases as K252a (Kase *et al.* 1987). However, it differs from K252a in a very important respect, namely that it is a very hydrophilic compound which does not freely pass through the cell membrane (Nagashima *et al.* 1991). Thus, it is to be expected that any effect of K252b on intact cells is due to inhibition of membrane protein kinases with an extracellular domain. From the protein kinases that are known to be inhibited by K252b, the tyrosine kinases, which are functional receptors for neurotrophic factors such as BDNF and NT-3, have an extracellular domain (Lindsay, Wiegand, Altar & DiStefano, 1994). Therefore, it is possible that the reducing effect of K252b on upregulated quantal contents at TIMG endplates, which was quite similar to that of K252a, was due to receptor tyrosine kinase inhibition.

Although K252b is probably the most selective receptor tyrosine kinase inhibitor known to date, we tried to obtain confirmation of involvement of protein tyrosine kinases in the mechanism underlying ACh upregulation in TIMG by testing the effect of the tyrosine kinase inhibitor tyrphostin 51. However, the compound lacked an effect on the stimulus-evoked ACh release, in contrast to the decreasing effect of K252a and K252b. However, it must be realized that so far, inhibition of the neurotrophin receptor tyrosine kinases by tyrphostin compounds has not been demonstrated unequivocally (Levitzki & Gazit, 1995).

It is possible that the increase of the spontaneous ACh release in TIMG, which was observed after incubation of muscles with tyrphostin 51, is due to inhibition of a non-receptor (cytoplasmic) tyrosine kinase. An interesting candidate is $pp60^{src}$, a tyrosine kinase that can be inhibited by tyrphostin compounds (O'Dell *et al.* 1991), and which phosphorylates synaptophysin, a synaptic vesicle protein that is part of the large protein complex involved in the docking of the vesicle to the presynaptic membrane and in the subsequent exocytosis of neurotransmitter (Walaas & Greengard, 1991). Another tyrosine protein kinase inhibitor used in the present study, K252a, does not have any inhibitory effect on $pp60^{src}$ (Tapley *et al.* 1992). Therefore, the present observation that K252a lacked an effect on the MEPP frequency does not contradict the idea of involvement of $pp60^{src}$ in the determination of MEPP frequency in TIMG. Hence, our results suggest that in TIMG the activity of at least two different tyrosine kinases is involved in spontaneous and evoked ACh release and that neurotrophin receptors and $pp60^{src}$ are possible candidates.

Next to its effect on MEPP frequency, tyrphostin 51 had a potentiating effect on MEPP amplitudes at TIMG endplates but not on those at control endplates. This was probably due to a postsynaptic effect of the kinase inhibitor on AChRs in TIMG. Although such a postsynaptic effect of tyrphostin 51, selective for TIMG, is an interesting observation, further discussion of this phenomenon falls beyond the scope of this paper.

It might be speculated that at TIMG endplates a neurotrophin is the retrograde signal that induces upregulation of ACh release at the nerve ending. This hypothesis is supported by a study that demonstrated a potentiating effect of the neurotrophic factors BDNF and NT-3 on ACh release at developing endplates *in vitro* (Lohof *et al.* 1993). Furthermore, it has been shown that embryonic and adult muscle cells are able to express mRNA for BDNF and NT-3 (Hohn, Leibrock, Bailey & Barde, 1990; Thoenen, Hughes & Sendtner, 1993) and that activity-induced release of NT-4 from muscle cells has a trophic effect on motor neurons (Funakoshi *et al.* 1995). With respect to the ACh release modulating aspect of BDNF and NT-3, it seems of interest that in the hippocampus an increase in BDNF mRNA takes place after the induction of LTP (Castrén *et al.* 1993) and that *in vitro* application of neurotrophins induces an enhancement of synaptic transmission that can be blocked by K252a (Kang & Schuman, 1995). Furthermore, BDNF, *in vitro*, enhances the release of ACh from synaptosomes obtained from cholinergic hippocampal neurons (Knipper, Berzaghi, Blöchl, Breer, Thoenen & Lindholm, 1994).

The question arises as to whether neurotrophin receptor tyrosine kinases are already present on normal nerve terminals of healthy animals, waiting to be activated by the neurotrophic factors that are released by the muscle fibre when the neuromuscular transmission is endangered. Experiments in which the *in vitro* effect of neurotrophins themselves are assessed on the quantal content at normal endplates could answer this question. The present observation that K252 compounds lack an effect on the quantal content of control muscles suggests that neurotrophin receptor tyrosine kinase activity under normal conditions is not involved in ACh release from motor nerve endings.

Benfenati, F. & Valtorta, F. (1993). Synapsins and synaptic transmission. *News in Physiological Sciences* **8**, 18–23.

Castrén, E., Pitkänen, M., Sirviö, J., Parsadanian, A., Lindholm, D., Thoenen, H. & Riekkinen, P. J. (1993). The induction of LTP increases BDNF and NGF mRNA but decreases NT3 mRNA in the dentate gyrus. *NeuroReport* **4**, 895–898.

Cheng, B., Barger, S. W. & Mattson, M. P. (1994). Staurosporine, K-252a, and K-252b stabilize calcium homeostasis and promote survival of CNS neurons in the absence of glucose. *Journal of Neurochemistry* **62**, 1319–1329.

Di Gregorio, F., Fesce, R., Cereser, S., Favaro, G. & Fiori, M. G. (1989). Spontaneous and nerve-evoked quantal transmission in regenerated motor terminals. *Cell Biology International Reports* **13**, 1119–1126.

Durant, N. N. & Lambert, J. J. (1981). The action of polymyxin B at the frog neuromuscular junction. *British Journal of Pharmacology* **72**, 41–47.

Eusebi, F., Molinaro, M. & Zani, B. M. (1985). Agents that activate protein kinase C reduce acetylcholine sensitivity in cultured myotubes. *Journal of Cell Biology* **100**, 1339–1342.

Funakoshi, H., Belluardo, N., Arenas, E., Yamamoto, Y., Casabona, A., Persson, H. & Ibanez, C. F. (1995). Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. *Science* **268**, 1495–1499.

Gazit, A., Yaish, P., Gilon, C. & Levitzki, A. (1989). Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. *Journal of Medicinal Chemistry* **32**, 2344–2352.

Greengard, P., Valtorta, F., Czernik, A. J. & Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **258**, 780–785.

Hashimoto, Y., Nakayama, T., Teramoto, T., Kato, H., Watanabe, T., Kinoshita, M., Tsukamoto, K., Tokunaga, K., Kurokawa, K., Nakanishi, S., Matsuda, Y. & Nonomura, Y. (1991). Potent and preferential inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by K252a and its derivative, KT5926. *Biochemical and Biophysical Research Communications* **181**, 423–429.

Hidaka, H. & Kobayashi, R. (1992). Pharmacology of protein kinase inhibitors. *Annual Review of Pharmacology and Toxicology* **32**, 377–397.

Hirsh, J. K., Silinsky, E. M. & Solsona, C. S. (1990). The role of cyclic AMP and its protein kinase in mediating acetylcholine release and the action of adenosine at frog motor nerve endings. *British Journal of Pharmacology* **101**, 311–318.

Hohn, A., Leibrock, J., Bailey, K. & Barde, Y.-A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339–341.

Hong, S. J. & Chang, C. C. (1989). Use of geographutoxin II (μ-conotoxin) for the study of neuromuscular transmission in mouse. *British Journal of Pharmacology* **97**, 934–940.

Kang, H. & Schuman, E. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* **267**, 1658–1662.

Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A. & Kaneko, M. (1987). K252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochemical and Biophysical Research Communications* **142**, 436–440.

Knipper, M., Berzaghi, M. D., Blöchl, A., Breer, H., Thoenen, H. & Lindholm, D. (1994). Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. *European Journal of Neuroscience* **6**, 668–671.

Knüsel, B. & Hefti, B. (1992). K-252 compounds: modulators of neurotrophin signal transduction. *Journal of Neurochemistry* **59**, 1987–1996.

Levitzki, A. & Gazit, A. (1995). Tyrosine kinase inhibition: an approach to drug development. *Science* **267**, 1782–1788.

Lindsay, R. M., Wiegand, S. J., Altar, C. A. & DiStefano, P. S. (1994). Neurotrophic factors: from molecule to man. *Trends in Neurosciences* **17**, 182–190.

Llinás, R., Gruner, J. A., Sugimori, M., McGuinness, T. L. & Greengard, P. (1991). Regulation by synapsin I and Ca^{2+}–calmodulin-dependent protein kinase II of transmitter release in squid giant synapse. *Journal of Physiology* **436**, 257–282.

Lohof, A. M., Ip, N. Y. & Poo, M. (1993). Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* **363**, 350–353.

McLachlan, E. M. & Martin, A. R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *Journal of Physiology* **311**, 307–324.

Mazzei, G. J., Katoh, N. & Kuo, J. F. (1982). Polymyxin B is a more selective inhibitor for phospholipid sensitive Ca^{2+}-dependent protein kinase than for calmodulin sensitive Ca^{2+}-dependent protein kinase. *Biochemical and Biophysical Research Communications* **109**, 1129–1133.

Nagashima, K., Nakanishi, S. & Matsuda, Y. (1991). Inhibition of nerve growth factor-induced neurite outgrowth of PC12 cells by a protein kinase inhibitor which does not permeate the cell membrane. *FEBS Letters* **293**, 119–123.

O'Dell, T. J., Kandel, E. R. & Grant, S. G. N. (1991). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Science* **353**, 558–560.

Oppenheim, R. W., Qin-Wei, Y., Prevette, D. & Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature* **360**, 755–759.

Pettit, D. L., Perlman, S. & Malinow, R. (1994). Potentiated transmission and prevention of further LTP by increased CaMKII activity in postsynaptic hippocampal slice neurons. *Science* **266**, 1881–1885.

Plomp, J. J. & Molenaar, P. C. (1994). The effect of some protein kinase inhibitors on the upregulation of acetylcholine release at endplates of α-bungarotoxin-treated rats. *Journal of Physiology* **479.P**, 2*P*.

Plomp, J. J., van Kempen, G. Th. H., De Baets, M. H., Graus, Y. M. F., Kuks, J. B. M. & Molenaar, P. C. (1995). Acetylcholine release in myasthenia gravis: regulation at single endplate level. *Annals of Neurology* **37**, 627–636.

Plomp, J. J., van Kempen, G. Th. H. & Molenaar, P. C. (1992). Adaptation of quantal content to decreased postsynaptic sensitivity at single endplates in α-bungarotoxin-treated rats. *Journal of Physiology* **458**, 487–499.

Plomp, J. J., van Kempen, G. Th. H. & Molenaar, P. C. (1994). The upregulation of acetylcholine release at endplates of α-bungarotoxin-treated rats: its dependency on calcium. *Journal of Physiology* **478**, 125–136.

Reuhl, T. O. K., Amador, M., Moorman, J. R., Pinkham, J. & Dani, J. A. (1992). Nicotinic acetylcholine receptors are directly affected by agents used to study protein phosphorylation. *Journal of Neurophysiology* **68**, 407–416.

Rosahl, T. W., Spillane, D., Missler, M., Herz, J., Selig, D. K., Wolff, J. R., Hammer, R. E., Malenka, R. C. & Südhof, T. C. (1995). Essential functions of synapsins I and II in synaptic vesicle regulation. *Nature* **375**, 488–493.

Silva, A. J., Stevens, C. F., Tonegawa, S. & Wang, Y. (1992). Deficient hippocampal long-term potentiation in α-calcium-calmodulin kinase II mutant mice. *Science* **257**, 201–206.

Tapley, P., Lamballe, F. & Barbacid, M. (1992). K252a is a selective inhibitor of the tyrosine protein kinase activity of the *trk* family of oncogenes and neurotrophin receptors. *Oncogene* **7**, 371–381.

Thoenen, H., Hughes, R. A., Sendtner, M. (1993). Trophic support of motoneurons: physiological, pathophysiological, and therapeutic implications. *Experimental Neurology* **124**, 47–55.

Van der Kloot, W. & Molgó, J. (1994). Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiological Reviews* **74**, 899–991.

Walaas, S. I. & Greengard, P. (1991). Protein phosphorylation and neuronal function. *Pharmacological Reviews* **43**, 299–349.

Wong, V., Arriaga, R., Ip, N. Y. & Lindsay, R. M. (1993). The neurotrophins BDNF, NT-3, and NT-4/5, but not NGF up-regulate the cholinergic phenotype of developing motor neurons. *European Journal of Neuroscience* **5**, 466–474.

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