Activation of protein kinase C potentiates postsynaptic acetylcholine response at developing neuromuscular synapses

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1 Phorbol 12-myristate 13-acetate (TPA, 1 μ M) and phorbol 12,13-dibutyrate (PDBu, 2 μ M), activators of protein kinase C (PKC), increased the mean amplitude and decay time of the spontaneous synaptic currents of *Xenopus* nerve-muscle coculture, whereas, 4 α -phorbol (2 μ M) which is an inactive phorbol analogue had no effect.

2 Staurosporine (0.5 μ M) and H-7 (10 μ M), inhibitors of PKC, inhibited the potentiation effects of TPA on the spontaneous synaptic currents.

3 Effects of TPA on the postsynaptic acetylcholine (ACh) sensitivity were examined by iontophoresis of ACh to the surface of embryonic muscle cells of 1-day-old *Xenopus* cultures. TPA increased both the amplitude and decay time of ACh-induced whole-cell currents in isolated myocytes.

4 TPA concentration-dependently increased the mean open time of low-conductance ACh channels but did not affect those of high-conductance ACh channels. PDBu but not 4 α -phorbol exhibited similar effects to TPA. Staurosporine and H-7 inhibited the increasing effects of TPA.

5 These results suggest that activation of PKC might be involved in synaptogenesis at developing neuromuscular synapses by the postsynaptic potentiation of ACh sensitivity.

Keywords: Phorbol 12-myristate 13-acetate (TPA); protein kinase C; ACh response; embryonic myocyte

Introduction

The characteristics of postsynaptic nicotinic acetylcholine (ACh) receptor channels determine the property of synaptic transmission at the neuromuscular junction. It has also been suggested that protein phosphorylation (one of the principal mechanisms of regulation of cellular metabolism) plays a major role in the regulation of synaptic function (Greengard, 1978), a fundamental event in signal transduction is receptor phosphorylation (Nestler & Greengard, 1984). Protein phosphorylation has been correlated with modulation of receptor activity by either enhancing or terminating the action of ligand (Hemmings *et al.*, 1989). The principal effect of adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent processes on muscle and electric organ AChRs has been reported to be an enhancement of the receptor phosphorylation as well as the rate of agonist-induced receptor desensitization (Huganir *et al.*, 1986; Middleton *et al.*, 1986; 1988; Mülle *et al.*, 1988). On the other hand, phorbol 12-myristate 13-acetate (TPA), an activator of protein kinase C, has also been reported to reduce ACh sensitivity in cultured myotubes (Eusebi *et al.*, 1985). The physiological role of desensitization or reduction of sensitivity of AChR remains unclear.

Recently, we have provided the first evidence that membrane permeable analogues of cyclic AMP and forskolin increased the ACh responses in the embryonic myocytes of *Xenopus* cell cultures (Fu & Poo, 1991). Furthermore, calcitonin gene-related peptide (CGRP) which is released from the nerve terminals and elevates postsynaptic cyclic AMP has also been shown to potentiate spontaneous synaptic currents and increase mean open time of ACh channels in *Xenopus* embryonic myocytes (Lu *et al.*, 1993). The involvement of cyclic AMP-dependent protein kinase (PKA) in the action of CGRP was implicated, since intracellular loading of a PKA inhibitor into the myocyte prevented the CGRP effects. In the present study, we examined further the effect of activation of protein kinase C (PKC) on the properties of ACh channels and the synaptic currents during the early phase of neuromuscular synaptogenesis. We found that agents activating PKC increased the synaptic currents and

iontophoretic ACh-induced currents in these embryonic myocytes. The present findings suggest that PKC and released trophic factors which activate PKC may be important in the physiological modulation of synaptic function during the early phase of synaptic development.

Methods

Culture preparations

Xenopus nerve-muscle cultures were prepared as previously described (Spitzer & Lamborghini, 1976; Anderson *et al.*, 1977; Sanes & Poo, 1989). Briefly, the neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos at stage 20–22 (Nieuwkoop & Faber, 1967) were dissociated in the Ca²⁺- and Mg²⁺-free Ringer solution supplemented with EDTA. The cells were plated on clean glass coverslips and were used for experiments after 24 h incubation at room temperature (20–22°C). The culture medium consisted of 50% (vol/vol) Ringer solution (composition mM: NaCl 115, CaCl₂ 2, KCl 2.5, HEPES 10, pH 7.6), 49% L-15 Leibovitz medium (Sigma), and 1% foetal bovine serum (GIBCO). Most of the experiments utilized the spherical myocytes in these cultures. Previous studies have shown that synapses formed between spinal neurones and these spherical myocytes have structural and functional properties similar to those observed *in vivo* (Evers *et al.*, 1989).

Electrophysiology

Whole-cell and cell-attached patch-clamp recording methods were similar to those described previously (Hamill *et al.*, 1981; Evers *et al.*, 1989). Whole-cell recording method was used in voltage-clamp configuration to monitor membrane currents induced by iontophoresis of ACh at isolated myocytes and synaptic currents at innervated myocytes. The solution inside the whole-cell recording pipettes contained mM: KCl 150, NaCl 1, MgCl₂ 1 and HEPES 10 (pH 7.2). Iontophoresis of ACh was applied to the surface of the myocyte by conventional glass microelectrodes filled with 3 M ACh

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Figure 1 Potentiation of synaptic activity by phorbol 12-myristate 13-acetate (TPA). (a) Continuous trace depicts membrane current measured by gigaohm-seal, whole-cell recording method from an innervated myocyte in 1-day-old *Xenopus* culture. The myocyte was voltage-clamped at -60 mV. Spontaneous synaptic currents (SSCs) appear as random downward deflection (filtered at 150 Hz). TPA was applied to the culture at time marked by the arrow. Samples of synaptic currents before and after drug application were superimposed and shown below the trace at higher time resolution (filtered at 10 kHz). Note the increase in the average amplitude and decay time of the synaptic currents after TPA treatment. Bars: 30 s, 170 pA and 3 ms, 150 pA for slow and fast traces, respectively. (b,c) Histograms of amplitude distribution for all SSC events before (b) and after (c) TPA application at the same synapse as in (a). (e,f) Histograms of decay time distribution for all SSC events before (e) and after (f) TPA application. (d,g) % cumulative frequency of amplitude (d) and decay time (g) distribution of all SSC events obtained from 4 cells. The difference between two sets of data obtained from control (○) and TPA-treated (●) is significant ($P < 0.05$, two-tail t test).

chloride (resistance 100–200 M Ω). The iontophoretic current of 2 ms duration was supplied by a Grass stimulator (SD9) through a microelectrode amplifier (Getting M5), which provided a braking current between 2 to 10 nA. For single channel recording, the patch pipettes were coated with Sylgard (Dow Corning) before polishing to reduce the capacitance of the electrode and the noise of the current recordings. The pipette was filled with Ringer solution containing low concentrations of ACh (1–5 nM). Single channel activities were recorded with a patch clamp amplifier (Dagan 8900) and the current signal was filtered at 3 kHz. The data were digitized by a digitizing unit (Neuro-Corder DR390) and stored on a videotape recorder for later playback on a storage oscilloscope (Tektronix 5113) and on oscillographic recorder (Gould RS3200) or for analysis by a microcomputer. For single channel analysis, the current signals were digitized at either 50 or 100 μ s intervals and analysed with PClamp

programme (Axon Inst.). The amplitude and duration of individual events were measured and stored in the computer. Events corresponding to opening of more than one channel were excluded from the open time analysis. Events with open time shorter than 500 μ s were not analysed because of possible attenuation and distortion. Open duration histograms were fitted with a single exponential and the amplitude histograms were fitted with Gaussian distribution curves, using least-squares method in both cases. All recordings were made at room temperature in culture medium. The results are expressed as the mean $\pm$ s.e.mean. The statistical significance was evaluated by Student's t test.

The following chemicals were used: acetylcholine (ACh), TPA (phorbol 12-myristate 13-acetate), PDBu (phorbol 12, 13-dibutyrate) and 4 α -phorbol (Sigma), staurosporine (Boehringer) and H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride) (Seikagaku Kogyo Co., Japan).

Results

Effects of protein kinase C (PKC) activators on spontaneous synaptic currents

In nerve-muscle cultures prepared from 1-day-old *Xenopus* embryos, synaptic contacts are established between dissociated spinal neurones and myotomal myocytes within the first day of culture. Spontaneous synaptic currents (SSCs) are readily detectable from the innervated myocytes by whole-cell voltage-clamp recording. These currents have been shown to be caused by spontaneous pulsatile ACh secretion from the neurone, since they were abolished by bath application of (+)-tubocurarine and unaffected by tetrodotoxin (Xie & Poo, 1986). Figure 1a depicts the recording of SSCs from an innervated myocyte before and after application of TPA (1 μ M). Bath application of TPA increased the frequency and the amplitude and decay time of the SSCs. The change in amplitude and decay time distribution was apparent on comparing the amplitude and decay time histograms of SSCs before and after exposure to TPA in the same cell (Figure 1b,c,e and f). Figure 1d and g show the cumulative frequency (%) obtained from 4 cells. TPA treatment shifted the curves

to the right. PDBu, another PKC activator, also increased both amplitude and decay time of the SSC currents, but 4 α -phorbol, an inactive phorbol analogue, had no effect (Figure 2). Figure 3 summarizes the results obtained with these phorbol ester analogues on the amplitude and decay time of the SSCs and also showed that staurosporine and H-7 (both PKC inhibitors), inhibited the potentiating effects of TPA. The increase in SSC frequency induced by TPA resulted from presynaptic potentiation of spontaneous ACh secretion. The increase in amplitude, on the other hand, could be caused by a higher amount of transmitter molecules in the ACh quanta or by an elevated sensitivity of the muscle cell membrane toward ACh. In the following experiment, we tested directly the effect of TPA on ACh sensitivity of embryonic myocytes.

Potentiation of muscle acetylcholine sensitivity by TPA

Gigaohm-seal, whole-cell recording of single non-innervated myocyte in 1-day-old *Xenopus* cultures was performed in voltage-clamp mode. Identical ACh pulses were applied repetitively by focal iontophoresis at the muscle surface, and the ACh-induced membrane currents were recorded. Figure 4

Figure 2 Effects of phorbol analogues on the spontaneous synaptic currents. Continuous traces in (a) and (d) depict membrane current measured by whole-cell recording from an innervated myocyte which was voltage-clamped at -60 mV. Spontaneous synaptic currents (SSCs) appear as random downward deflections. Samples of synaptic currents were shown below at higher time resolution. Note that phorbol 12,13-dibutyrate (PDBu) (a) but not 4 α -phorbol (d) increased the amplitude and decay time of the synaptic currents. Bars: 30 s, 160 pA and 3 ms, 150 pA for slow and fast traces, respectively. (b,e and f) % cumulative frequency of amplitude (b,e) and decay time (c,f) distribution of all SSC events obtained from 4 cells. The difference between two sets of data points obtained from control (○) and PDBu-treated (●) is significant ($P < 0.05$, two-tail t test). There is no significant difference between control (○) and 4 α -phorbol-treated (●) groups ($P > 0.05$).

Figure 3 Effects of protein kinase C activation on the spontaneous synaptic currents. Phorbol 12-myristate 13-acetate (TPA, $1 \mu\text{M}$) and phorbol 12,13-dibutyrate (PDBu, $2 \mu\text{M}$) which are protein kinase C activators increased the amplitude and decay time of the spontaneous synaptic currents, but 4α -phorbol ($2 \mu\text{M}$), an inactive phorbol analogue, had no effect. Staurosporine ($0.5 \mu\text{M}$) and H-7 ($10 \mu\text{M}$), both protein kinase C inhibitors, inhibited the potentiation effects of TPA. ($n = 5-8$).

Figure 4 Effects of phorbol 12-myristate 13-acetate (TPA) on the acetylcholine (ACh) sensitivity of isolated myocyte. Identical iontophoretic pulses of ACh were applied to the surface of an isolated myocyte in a 1-day-old *Xenopus* culture, and the membrane current of the myocyte was recorded by whole-cell voltage-clamp recording ($V_c = -60 \text{ mV}$). Oscilloscopic traces of 10 superimposed consecutive ACh-induced currents of control (a) and TPA-treated (b) are shown below. Note the increase in the amplitude and decay time of the membrane currents 30 min after TPA ($1 \mu\text{M}$) treatment. Scale bars: 8 s, 320 pA and 10 ms, 200 pA for slow and fast traces, respectively.

depicts the recording of ACh-induced membrane currents before and 30 min after TPA application. Similar to the effects of TPA on synaptic currents, both the peak amplitude and the decay time of ACh-induced currents were significantly increased. The mean amplitude and decay time of ACh-induced current after TPA treatment was $198.1 \pm 20.8\%$ and $149.0 \pm 19.6\%$ ($n = 9$) that of the control (prior to the TPA treatment), respectively.

Figure 5 Effects of phorbol 12-myristate 13-acetate (TPA) on the properties of single acetylcholine (ACh) channel. Recordings were made at $+60 \text{ mV}$ applied potential from cell-attached patches. The pipette was filled with Ringer containing 1 nM of ACh. (a) Samples of recordings of single ACh channel currents before (left panel) and after (right panel) bath application of $1 \mu\text{M}$ TPA were obtained from different cell-attached patches of the same myocyte. Bars: 7 ms and 8 pA. (b) and (c) show the mean open time of low- and high-conductance ACh channels before (left panels) and after (right panels) application of $1 \mu\text{M}$ TPA.

Effects of protein kinase C activators on single ACh channel

Single channel recordings were made on isolated myocyte to investigate the mechanism of action of TPA on the muscle membrane ACh sensitivity. Cell-attached patch recording of ACh channels with micropipettes containing $1-5 \text{ nM}$ ACh was used to examine the ACh channel properties before and after extracellular exposure of the myocyte to TPA. Figure 5a shows samples of single channel events observed before and after TPA exposure from different patches of the same myocyte when myocyte was hyperpolarized $+60 \text{ mV}$ from rest. ACh channels on these embryonic muscle cells can be distinguished as two major types, low- and high-conductance channels; the current amplitudes were $5.07 \pm 0.04 \text{ pA}$ and $7.51 \pm 0.05 \text{ pA}$ ($n = 6$), respectively. We found that the amplitude of single channel currents for both types of ACh channels remained unchanged after TPA treatment ($5.09 \pm 0.02 \text{ pA}$ and $7.52 \pm 0.04 \text{ pA}$ for low- and high-conductance ACh channels, respectively, $n = 6$). However, the mean channel open time of the low-conductance but not high-conductance ACh channels was increased after TPA treatment (Figure 5b and c). TPA concentration-dependently increased the mean open time of low-conductance ACh channels (Figure 6). The involvement of protein kinase C activation as the mechanism of action of TPA was further supported by the inhibitory effect of staurosporine and H-7 (both PKC inhibitors) on the action of TPA (Figure 7). Furthermore, PDBu but not 4α -phorbol increased the mean open time of low-conductance ACh channels (Figure 7).

Figure 6 Concentration-dependent increase of mean open time of single acetylcholine (ACh) channel by phorbol 12-myristate 13-acetate (TPA). Each data point is obtained from at least 7 patches at each concentration. Note that TPA concentration-dependently increased the mean open time of low-conductance (○) but not high-conductance (●) ACh channels.

Figure 7 Effects of protein kinase C activation on the mean open time of single acetylcholine (ACh) channel. Phorbol 12-myristate 13-acetate (TPA, 1 μM) and phorbol 12, 13-dibutyrate (PDBu, 2 μM) which are protein kinase C activators increased the mean open time of low-conductance (open columns) but not high-conductance (hatched columns) ACh channels, but 4α-phorbol (2 μM), an inactive phorbol analogue, had no effect. Staurosporine (0.5 μM) and H-7 (10 μM), both protein kinase C inhibitors, inhibited the increasing effect of TPA.

Discussion

The characteristics of postsynaptic nicotinic acetylcholine (ACh) channels determine the property of synaptic transmission at the neuromuscular junction. Phosphorylation reactions are known to be able to modulate synaptic transmission both pre- and post-synaptically (Kennedy, 1983; Nestler *et al.*, 1984). Phosphorylation of the muscle ACh receptor (AChR) has been implicated in playing a role in the regulation of the receptor ion channelling. Cyclic AMP-elevating agents, which act presumably by activating cyclic AMP-dependent protein kinase (PKA) were shown to enhance receptor desensitization (Albuquerque *et al.*, 1986; Middleton *et al.*, 1986). On the other hand, many important phosphorylation reactions are mediated by protein kinase C (PKC) (Nishizuka, 1986; Huganir & Greengard, 1987; Nishizuka, 1988), which can be specifically activated by the phorbol ester, TPA. TPA was shown to enhance spontaneous ACh secretion in this experiment, in accordance with previous reports showing that TPA enhanced neurotransmitter release presynaptically at motor nerve terminals (Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Murphy & Smith, 1987; Shapira *et al.*, 1987; Caratsch *et al.*, 1988). Although there is growing evidence that PKC can modulate the activity of voltage-dependent Ca^{2+} , Cl^- and K^+ conductance (Kacz-

marek, 1987; Shearman *et al.*, 1989), reports of effects of PKC on ligand-gated channels are rare. Recently, it was reported that the effects of PKC on the postsynaptic sensitivity of AChR showed species differences. TPA causes a transient decrease in ACh sensitivity at the junctional AChRs of adult frog muscle fibres (Caratsch *et al.*, 1986) and a sustained decrease in ACh sensitivity accompanied by a decrease in both channel conductance and opening probability at the extrajunctional AChRs of embryonic chick myotubes (Eusebi *et al.*, 1985; 1987). On the other hand, TPA causes an early decrease followed by a delayed increase and again a decrease to below pretreatment level in rat skeletal muscle (Caratsch *et al.*, 1989). In the present study, we obtained evidence that activation of PKC increases postsynaptic ACh responses in embryonic myocytes.

In this work, we have demonstrated that TPA and PDBu potentiate ACh responses in the embryonic myocyte of 1-day-old *Xenopus* culture. The involvement of PKC in this effect is further supported by the inhibitory effect on the action of TPA of staurosporine and H-7 (both PKC inhibitors) together with the lack of potentiating effect of 4α-phorbol, which does not activate PKC (Castagna *et al.*, 1982). Developmental changes of ACh receptor channels have been studied extensively in *Xenopus* muscle cells *in vitro* (Brehm *et al.*, 1984a; Rohrbough & Kidokoro, 1990) as well as *in vivo* (Kullberg *et al.*, 1981; Brehm *et al.*, 1984b; Owens & Kullberg, 1989). Shortly after the initial insertion of ACh receptor channels, the majority of channels are an embryonic type (low-conductance channels) which have a prolonged mean open time and lower conductance. The adult-type channels (high-conductance channels) are rare at the early stages but they are detectable from the beginning. They have about 50% greater unitary conductance than embryonic-type channels and shorter mean open time. During development, the relative population of the adult-type channels increases (Kidokoro & Gruener, 1982; Brehm *et al.*, 1984a). The results of this study demonstrate that application of TPA increased the amplitude and decay time of iontophoretic ACh-induced whole-cell current. Measurement of single ACh channel activity was consistent with this result. The open duration and opening probability of low-conductance ACh channels but not those of high-conductance channels were increased by the application of TPA. These potentiating effects of PKC on ACh channels of *Xenopus* embryonic myocytes appear very similar to those observed in PKA modulation (Fu & Poo, 1991). It is possible that the muscle cells used in previous studies may have reached a more mature stage of development and their ACh channels lost their susceptibility to potentiation, as we found for older *Xenopus* myocytes (unpublished data). *In vitro* studies indicate that all four subunits of the AChR from *Torpedo* electric organ and rat muscle can be phosphorylated by several kinases (Huganir & Greengard, 1983; Huganir *et al.*, 1984; Steinbach & Zempel, 1987). PKA phosphorylates the α and δ subunits, PKC phosphorylates the α and δ subunits. Their results show that the rate of desensitization was enhanced for the phosphorylated AChRs. It remains to be determined whether the potentiation of AChR response by PKC in *Xenopus* embryonic myocytes is mediated by the phosphorylation of the receptor subunits and which phosphorylated subunits are responsible for this potentiating action.

Spontaneous synaptic activity begins soon after nerve-muscle contact. This early activity is known to be responsible for inducing spontaneous muscle contractions during the early phase of synaptogenesis. Kidokoro & Saito (1988) have found that spontaneous synaptic activity is responsible for the accelerated development of striation in the innervated myocyte. Maturation of synaptic specialization could also be influenced by the presence of the activity and the increase of intracellular Ca^{2+} concentration through the influx from AChR channels. Thus this work provides an indication that extracellular agents such as primary neurotransmitters, co-transmitters or hormones which are capable of activating

PKC, may regulate synaptic ion channels and synaptic maturation at embryonic developing neuromuscular synapses.

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References

- ALBUQUERQUE, E.X., DESHPANDE, S.S., ARACAVA, Y., ALKONDON, M. & DALY, J.W. (1986). A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. *FEBS Lett.*, **199**, 113–120.
- ANDERSON, M.J., COHEN, M.W. & ZORYCHIA, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.*, **268**, 731–756.
- BREHM, P., KIDOKORO, Y. & MOODY-CORBETT, F. (1984a). Acetylcholine receptor channel properties during development of *Xenopus* muscle cells in culture. *J. Physiol.*, **357**, 203–217.
- BREHM, P., KULIBERG, R. & MOODY-CORBETT, F. (1984b). Properties of non-junctional acetylcholine receptor channels in innervated muscle of *Xenopus laevis*. *J. Physiol.*, **350**, 631–648.
- CARATSCH, C.G., GRASSI, F., MOLINARO, M. & EUSEBI, F. (1986). Postsynaptic effects of the phorbol ester TPA on frog end-plates. *Pflügers Arch.*, **407**, 409–413.
- CARATSCH, C.G., KNOFLACH, F., GRASSI, F. & EUSEBI, F. (1989). Regulation of acetylcholine receptor function by the phorbol ester TPA in rat skeletal muscle. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 82–86.
- CARATSCH, C.G., SCHUMACHER, S., GRASSI, F. & EUSEBI, F. (1988). Influence of protein kinase C-stimulation by a phorbol ester on neurotransmitter release at frog end-plates. *Naunyn-Schmied. Arch. Pharmacol.*, **337**, 9–12.
- CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, Y. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.
- EUSEBI, F., GRASSI, F., MOLINARO, M. & ZANI, B.M. (1987). Acetylcholine regulation of nicotinic receptor channels through a putative G protein in chick myotubes. *J. Physiol.*, **393**, 635–645.
- EUSEBI, F., MOLINARO, M. & CARATSCH, C.G. (1986). Effects of phorbol ester on spontaneous transmitter release at frog neuromuscular junction. *Pflügers Arch.*, **406**, 181–183.
- EUSEBI, F., MOLINARO, M. & ZANI, B.M. (1985). Agents that activate protein kinase C reduce acetylcholine sensitivity in cultured myotubes. *J. Cell Biol.*, **100**, 1339–1342.
- EVERS, J., LASER, M., SUN, Y., XIE, Z. & POO, M.M. (1989). Studies of nerve-muscle interactions in *Xenopus* cell culture: analysis of early synaptic currents. *J. Neurosci.*, **9**, 1523–1539.
- FU, W.-M. & POO, M.M. (1991). Modulation of acetylcholine channel by DBcAMP in *Xenopus* cell culture. *Soc. Neurosci.*, **17**, 1096 (Abstr).
- GREENGARD, P. (1978). Phosphorylated proteins as physiological effectors. *Science*, **199**, 146–152.
- HAIMANN, C., MELDOLESI, J. & CECCARELLI, B. (1987). The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, enhances the evoked quanta release of acetylcholine at the frog neuromuscular junction. *Pflügers Arch.*, **408**, 27–31.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.
- HEMMINGS, H.C., NARIN, A.C., MCGUINNESS, T.L., HUGANIR, R.L. & GREENGARD, P. (1989). Role of protein phosphorylation in neuronal signal transduction. *FASEB J.*, **3**, 1583–1592.
- HUGANIR, R.L., DELCOUR, A.H., GREENGARD, P. & HESS, G.P. (1986). Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. *Nature*, **321**, 774–776.
- HUGANIR, R.L. & GREENGARD, P. (1983). cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1130–1134.
- HUGANIR, R.L. & GREENGARD, P. (1987). Regulation of receptor function by protein phosphorylation. *Trends Pharmacol. Sci.*, **8**, 472–477.
- HUGANIR, R.L., MILES, K. & GREENGARD, P. (1984). Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 6968–6972.
- KACZMAREK, L.K. (1987). The role of protein kinase C in the regulation of ion channels and neurotransmitter release. *Trends Neurosci.*, **10**, 30–34.
- KENNEDY, M.B. (1983). Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal function. *Annu. Rev. Neurosci.*, **6**, 493–525.
- KIDOKORO, Y. & GRUENER, R. (1982). Distribution and density of α -bungarotoxin binding sites on innervated and noninnervated *Xenopus* muscle cells in culture. *Develop. Biol.*, **91**, 78–85.
- KIDOKORO, Y. & SAITO, M. (1988). Early cross-striation formation in twitching *Xenopus* myocytes in culture. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1978–1982.
- KULLBERG, R., BREHM, P. & STEINBACH, J.H. (1981). Nonjunctional acetylcholine receptor channel open time decreases during development of *Xenopus* muscle. *Nature*, **289**, 411–413.
- LU, B., FU, W.-M., GREENGARD, P. & POO, M.M. (1993). CGRP enhances nicotinic ACh channel activity at developing neuromuscular synapses through cAMP-dependent protein kinase. *Nature*, **363**, 76–79.
- MIDDLETON, P., JARAMILLO, F. & SCHUETZE, S.M. (1986). Forskolin increases the rate of acetylcholine receptor desensitization at rat soleus endplate. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4967–4971.
- MIDDLETON, P., RUBIN, L.L. & SCHUETZE, S.M. (1988). Desensitization of acetylcholine receptors in rat myotubes is enhanced by agents that elevate intracellular cAMP. *J. Neurosci.*, **8**, 3405–3412.
- MULLE, C., BENOIT, P., PINSET, C., ROA, M. & CHANGEUX, J.-P. (1988). Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 5728–5732.
- MURPHY, R.L.W. & SMITH, M.E. (1987). Effects of diacylglycerol and phorbol ester on acetylcholine release and action at the neuromuscular junction in mice. *Br. J. Pharmacol.*, **90**, 327–334.
- NESTLER, E.J. & GREENGARD, P. (1984). *Protein Phosphorylation in the Nervous System* (New York: Wiley).
- NESTLER, E.G., WALAAS, S.I. & GREENGARD, P. (1984). Neuronal phosphoprotein: physiological and clinical implications. *Science*, **225**, 1365–1370.
- NIEUWKOOP, P.D. & FABER, J. (1967). *Normal Table of Xenopus laevis*. Second Edition. Amsterdam: North Holland.
- NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**, 305–312.
- NISHIZUKA, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, **334**, 661–665.
- OWENS, J.L. & KULLBERG, R. (1989). In vivo development of nicotinic acetylcholine receptor channel in *Xenopus* myotomal muscle. *J. Neurosci.*, **9**, 1018–1028.
- ROHRBOUGH, J. & KIDOKORO, Y. (1990). Changes in kinetic of acetylcholine receptor channels after initial expression in *Xenopus* myocyte culture. *J. Physiol.*, **425**, 245–269.
- SANES, D.H. & POO, M.M. (1989). In vitro analysis of position- and lineage dependent selectivity in the formation of neuromuscular synapse. *Neuron*, **2**, 1237–1244.
- SHAPIRA, R., SILBERBERG, S.D., GINSBURG, S. & RAHAMIMOFF, R. (1987). Activation of protein kinase C augments evoked transmitter release. *Nature*, **325**, 58–60.
- SHEARMAN, M.S., SEKIGUCHI, K. & NISHIZUKA, Y. (1989). Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol. Rev.*, **41**, 211–237.
- SPITZER, N.C. & LAMBORGHINI, J.C. (1976). The development of the action potential mechanism of amphibian neurons isolated in culture. *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1641–1645.
- STEINBACH, J.H. & ZEMPEL, J. (1987). What does phosphorylation do for the nicotinic acetylcholine receptor? *Trends Neurosci.*, **10**, 61–64.
- XIE, A. & POO, M.M. (1986). Initial events in the formation of neuromuscular synapse. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7069–7073.

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