Agents That Activate Protein Kinase C Reduce Acetylcholine Sensitivity in Cultured Myotubes

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ABSTRACT We have examined acetylcholine (ACh)-elicited potentials or currents in current- or voltage-clamped cultured myotubes exposed to 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a potent tumor promoter that activates protein kinase C. Although this agent had little action on either membrane resting potential or electrical resistance, a reversible decrease in ACh sensitivity was induced on 3–4-d-old chick myotubes. Depression of transmitter action by TPA was extended to 7–8-d mouse myotubes only when they were treated with phosphatidylserine. Glyceryl dioleate had effects on myotubes similar to those of TPA but with a reduced efficacy. We conclude that the activation of protein kinase C might be involved with the capacity of ACh receptors to respond to transmitter stimulation.

Acetylcholine (ACh) receptors located on specialized membrane regions of innervated muscle fibers and on entire surface of denervated fibers or cultured myotubes become desensitized in the continued presence of the transmitter, owing to their inactivation (1). However, the molecular mechanisms underlying this decrease in membrane chemosensitivity are still under question (2). Several recent findings indicate tight connections between the function of membrane ionic channels and protein phosphorylation (3). In the present study we have examined the action of ACh on chick and mouse cultured myotubes in the presence of the tumor promoting phorbo1 ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a known activator of the calcium- and phospholipid-dependent protein kinase C (PrkC) (4–7). We show here a reversible decrease in the extent of ACh-induced depolarizations and an enhancement of the rate of AChR desensitization of myotubes exposed to TPA (10−6–10−8 M). Thus, this study, in addition to its biological interest, might give some clues to the understanding of the mechanism of receptor desensitization itself.

MATERIALS AND METHODS

We prepared primary cultures of muscle cells from embryonic chick muscle and from mouse (C57) adult muscle, as described earlier (8, 9). Electrophysiological recordings were made in cylindrical myotubes with a 20–50-μm diam and an apparent length of ~200–300 μm, as described fully elsewhere (10–12). Iontophoretic pulses of ACh were applied to a site ~20 μm from the recording pipette. We applied at least three different doses of ACh to each spot on a myotube surface to obtain the largest response with a small ACh current. Chemosensitivity, conventionally expressed as peak of depolarization versus the charge passed through the ACh electrode (13), was calculated from data of the linear part of the dose–response curve (10). Since depolarization depends on various membrane parameters (14), all values of chemosensitivity were corrected and referred to mean resting potential and inversion potential (15, 16). Cultures kept at 37°C by irradiating infrared light were equilibrated in standard minimum essential medium (MEM) buffered with 10 mM HEPES/NaOH (bath volume, 1.5 ml). TPA, and in some cases ACh (as indicated in the text), was applied by perfusion or added to the bathing fluid from a stock solution. The TPA stock solution was 1 mM in 1% dimethyl sulfoxide. Dimethyl sulfoxide was ineffective in altering ACh sensitivity in the range 0.01–0.1%, the same used in the experiments. Phosphatidylserine (Ptd-Ser) and glyceryl dioleate (Gl-dio) were applied in a bath after suspension in MEM by sonication for 2–3 min at 4°C. All substances used were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS AND DISCUSSION

Myotubes from 3–4-d primary cultures of chick embryo myogenic cells showed a resting potential of −44.3 ± 1.6 mV (mean ± SEM; n = 95) after a single electrode penetration. This value did not decrease significantly when a second electrode was inserted into the cell, and it remained stable for 2 to 3 h. When cells were exposed to the potent tumor promoter TPA (10−6–10−8 M), which recognizes binding sites on the cell membrane (for review see references 17 and 18) and induces dedifferentiation effects in chick myotubes (19), the amplitude of their response to ionophoretic ACh exhibited a detectable decrease within 2–4 min. The decline of the ACh depolarization in the presence of TPA was maximal after 30–50 min and concomitant with a decrease in absolute membrane potential. This decrease was too small to account for the decline in ACh response. The membrane input resistance (Rm) and equilibrium potential of ACh response (−4.8 ± 0.6;
n = 7) remained substantially unchanged. The transmitter sensitivity determined conventionally as potential change/ 
ACh charge (12) was as low as 60% of the resting value in 
some myotubes, 10% in others, after 30 min of TPA perfu-
sion. Membrane potential and ACh sensitivity recovered 
promptly when the tumor promoter was removed from the 
bathing fluid. The recovery was complete when the myotube 
exposure did not exceed 60-80 min. TPA concentrations of 
>10^{-6} M were not tested. The 

TPA application, with same time course as ACh-sensitivity. Membrane potential and ACh sensitivity recovered 
to the effect seen in chick myotubes.

Recent studies suggest that PrkC has a binding site for TPA (for review see reference 22), as the tumor promoter phorbol 
ester directly activates the enzyme both in vivo and in vitro. 
In in vitro PrkC activation both Ptd-Ser and calcium ions are 
dispensable (23). When mouse myotubes equilibrated in 
MEM containing Ptd-Ser (100 #g/ml) were successively ex-
posed to TPA (10^{-6}-10^{-4} M), their ACh sensitivity declined 
by 80-90% within 30 min, whereas their membrane potential, 
input resistance, and equilibrium potential for ACh response 
(-3.4 ± 0.9 mV; n = 7) remained substantially stable (see 
Table I and Fig. 2). TPA was ineffective in lowering ACh 
sensitivity when myotubes were treated with phosphatidyl-
choline, a phospholipid known to inhibit PrkC activation 
(23). In contrast to chick myotube, which exhibited a per-
manent decrease in ACh sensitivity during TPA exposure. 
ACh sensitivity of the mouse myotube after a transient decline 
partially recovered within 20-60 min even in the presence of 
the agent (Fig. 2). ACh sensitivity was reduced again in a 
subsequent exposure to TPA after a new treatment with Ptd-
Ser and recovered completely after washing was done.

During prolonged application of ACh, muscle cells become 
unresponsive (24). This is also true of cultured chick myotubes 
but not of rat myotubes (25). Bath application of ACh (10^{-5} 
M) depolarized the mouse myotubes to their reversal poten-
tial, which was ~-5 mV in MEM as mentioned above. In 
contrast to rat myotubes, which do not exhibit desensitization 
(25), the ACh-induced depolarization in the mouse myotube 
could be sustained for periods as long as 3-5 min, and 
membrane potential reverted to original values within 5-10 
min after ACh bath application. For myotubes equilibrated 
in MEM containing Ptd-Ser (100 #g/ml) and TPA (10^{-7} M) 
desensitization was more pronounced, and the membrane 
potential recovered to normal within 10-70 s after ACh bath 
application (10^{-7} M). Repetitive iontophoretic ACh applica-
tions (0.5-2 s, 10-50 nA, 0.1 Hz) could not desensitize ACh 
receptors in untreated myotubes. A degree of desensitization 
was induced by equivalent ACh applications in Ptd-Ser, TPA-
treated myotubes (Fig. 2).

When diacylglycerol, which is a physiological activator of 
PrkC (26), was substituted for TPA in its unsaturated form 
(Gl-dio), it was less potent in reducing ACh sensitivity of the 
mouse Ptd-Ser-treated myotube with respect to TPA. In fact, 
Gl-dio concentrations of 3-7 x 10^{-5} M were required to 
reduce the myotube ACh sensitivity, and the effect was not 
as pronounced as with TPA added at 10^{-7} M (e.g., Fig. 3). 
For instance, in 12 cells examined ACh sensitivity was 16.5 
± 4.8 vs 3.2 ± 0.6 mV/nC determined after TPA application 
(Table I). The same result was obtained when chick myotubes 
not treated with Ptd-Ser were equilibrated in MEM and 
exposed to Gl-dio (7 x 10^{-5} M). Gl-dio had no effect at 
concentrations < 2 x 10^{-5} M. Since Gl-dio and Ptd-Ser are

**Table 1**

|                  | Control          | TPA              |
|------------------|------------------|------------------|
|                  | RP   | Rin | σ       | RP   | Rin | σ       |
|                  | mV   | mΩ  | mV/nC  | mV   | mΩ  | mV/nC  |
| 4-d-old chick myotube | -43.9 ± 1.9 (57) | 19 ± 3 (24) | 49 ± 4.3 (54) | -31.1 ± 1.5 (27) | 16 ± 2 (21) | 23.3 ± 4.1 (32) |
| 7-d-old Ptd Ser-treated mouse myotube | -39.6 ± 1.7 (31) | 25 ± 4 (10) | 27.8 ± 4.2 (24) | -41.1 ± 1.1 (27) | 23 ± 3 (10) | 3.2 ± 0.6 (18) |

RP, membrane resting potential; Rin, membrane input resistance; σ, ACh sensitivity. Number of observations is in brackets.
Concentrations of > 10^{-6} M were not tested. Before could not induce any electrical change on myotubes. TPA (10^{-6}-10^{-8} M) induced, in the chick myotube, a reversible phorbol-12,13-didecanoate at a concentration as low as 10^{-6}-10^{-7} M to activate the enzyme (4, 29). The application of 4-alpha-analogue of TPA inactive in promoting tumors and unable to activate the enzyme (4, 29). The application of 4-alpha-phorbol-12,13-didecanoate at a concentration as low as 10^{-6}-10^{-7} M under the same experimental conditions as described before could not induce any electrical change on myotubes. Concentrations of >10^{-6} M were not tested.

The results reported here show that the tumor promoter TPA (10^{-6}-10^{-8} M) induced in the chick myotube a reversible decrease in its receptor-mediated chemo-sensitivity. This effect is extended to the mouse myotube in the stringent presence of Ptd-Ser, but it fails to occur when TPA is substituted with 4-alpha-phorbol-12,13-didecanoate, or Ptd-Ser with phosphatidylycholine. Unsaturated diacylglycerol acts on myotubes in a way similar to TPA but with a markedly reduced potency. Since in other in vivo and in vitro systems PrkC is activated under the same experimental conditions that we have used (22, 26), the main conclusion of the present report is that PrkC activation is involved in some way, either directly or indirectly, with either the production in the number of functional ACh receptors or their capacity to respond to ACh stimulation. It is known that muscarinic-cholinergic agonists induce a rapid hydrolysis of polyphosphoinositides in the plasma membrane of their target cells to yield diacylglycerol, a potent activator of PrkC (22, 26, 27). It remains to be seen whether analogous biochemical processes underlying protein phosphorylation, which may be involved in transmitter-sensitivity decline, are induced by ACh at the nicotinic-cholinergic receptors.

As mentioned before, mouse myotubes are unresponsive to tumor promoters, and we have shown here that TPA was ineffective on mouse myotubes unless in the presence of Ptd-Ser. Since an approximate correlation is described between the ability of TPA to promote tumors and to activate PrkC (4), tumor promotion might be somehow regulated by the composition and the organization of membrane lipids indispensible in PrkC activation.

The suggestion from the present data that a decrease in transmitter sensitivity of some muscle diseases (10, 30, 31) might be mediated by an increased phosphoinositide turnover is intriguing idea worthy of further study.

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