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)[1] 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 phorbol 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 dedifferentiative 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 (R_{in}) and equilibrium potential of ACh response (−4.8 ± 0.6;

[1] *Abbreviations used in this paper*: ACh, acetylcholine; Gl-dio, glyceryl dioleate; MEM, minimum essential medium; PrkC, protein kinase C; Ptd-Ser, phosphatidylserine; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

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 perfusion. 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 $<10^{-8}$ M were ineffective in lowering ACh-induced depolarizations. TPA concentrations of $>10^{-6}$ M were not tested. The membrane current induced by the action of ACh in voltage-clamped myotubes declined progressively by 50–70% during TPA application, with same time course as ACh-sensitivity. Fig. 1 and Table I give a summary of the results.

As mentioned before, TPA is effective in inducing dedifferentiative alterations in developing chick myotubes at a concentration as low as 10^{-7} M (19) but ineffective in promoting equivalent morphological and biochemical changes in cultured mouse myotubes (20, 21). Application of TPA (10^{-6}–10^{-8} M) on mouse myotubes was ineffective in lowering transmitter sensitivity and membrane potential, in contrast

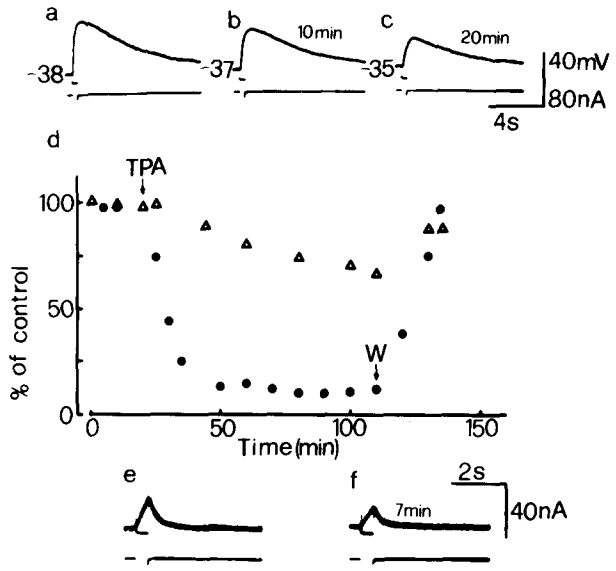

FIGURE 1 The effect of bath-applied TPA (10^{-7} M) on the ACh response of three different 4-d-old chick myotubes. (a–c) ACh depolarization produced by iontophoresis to the surface of a cell before (*a*) and after (*b* and *c*) TPA exposure at indicated times. Values next to traces indicate membrane potential. (*Top*) Potential; (*bottom*) iontophoretic current. (*d*) The time course of changes in membrane potential (triangles) and ACh sensitivity (circles) measured at a myotube after TPA application. Arrows indicate bath application of the drug and washing (*W*). (e and *f*) The membrane current activated by ACh before (e) and after (*f*) TPA exposure at indicated time. Holding potential, −50 mV. (*Top*) Membrane current; (*bottom*) iontophoretic current.

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 indispensable (23). When mouse myotubes equlibrated in MEM containing Ptd-Ser (100 μg/ml) were successively exposed to TPA (10^{-6}–10^{-8} 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 phosphatidylcholine, a phospholipid known to inhibit PrkC activation (23). In contrast to chick myotube, which exhibited a permanent 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 potential, 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 μg/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^{-5} M). Repetitive iontophoretic ACh applications (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 × 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 × 10^{-5} M). Gl-dio had no effect at concentrations < 2 × 10^{-5} M. Since Gl-dio and Ptd-Ser are

TABLE I

Effects of TPA (10^{-7} M) on Electrical Parameters of Cultured Myotubes after 30 min of Drug Exposure

	Control			TPA		
	RP	R_{in}	σ	RP	R_{in}	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; R_{in}, membrane input resistance; σ, Ach sensitivity. Number of observations is in brackets.

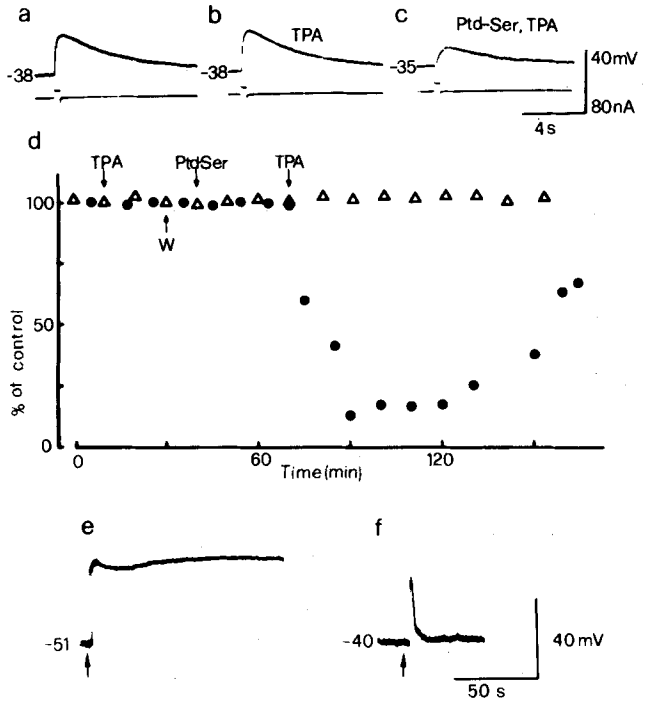

FIGURE 2 The effect of bath-applied TPA (10^{-7} M) on the ACh response of five different mouse myotubes. (a–c) ACh depolarization produced by iontophoresis of ACh at a 7-d-old myotube: (*a*) control; (*b*) 10 min of TPA-exposure; (c) 10 min of TPA-exposure after treatment with Ptd-Ser (100 μg/ml). The top trace is membrane potential; iontophoretic current is on the bottom. Numbers next to traces are membrane resting potentials. (*d*) The time course of changes in ACh sensitivity induced by the agent at a 8-d-old myotube. Notice a decreased ACh-sensitivity only when cell was treated with Ptd-Ser (100 μg/ml) and its partial recovery during the exposure to TPA. Arrows as in Fig. 1. Resting membrane potential, −41 mV; resting ACh sensitivity, 21 mV/nC. (e) ACh depolarization produced by bath-application of ACh (10^{-5} M) in an untreated 8-d-old myotube. Membrane potential recovered within the next 7 min. Number in near potential trace, membrane resting potential; arrow, ACh application. (*f*) Fast desensitization induced in a Ptd-Ser-, TPA-treated 8-d-old myotube (10 min of treatment) by bath application of ACh (10^{-5} M). Number next to potential trace, membrane potential.

water insoluble, in our system it is difficult to translate the concentrations we used into physiological terms. It is known that diacylglycerol is transiently produced in membrane as a consequence of extracellular signals, and its appearance is always associated with activation of PrkC (22, 27). Thus, the apparent reduced efficacy of Gl-dio as compared with that of TPA might be related to its rapid degradation in the cell membrane, as it occurs under physiological conditions, or to lack of uptake by the myotubes (due for instance to its inability to intercalate into the membrane [28]).

In some experiments chick myotubes from 3–4-d primary cultures or mouse 7–8-d myotubes treated with Ptd-Ser (100 μm/ml) were exposed to 4-alpha-phorbol-12,13-didecanoate, an 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

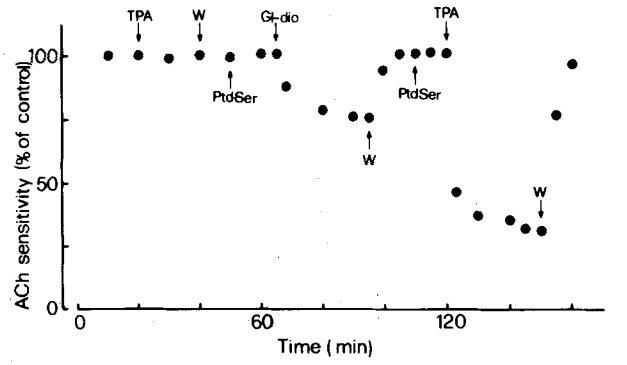

FIGURE 3 The time course of changes in ACh-sensitivity induced by Gl-dio (3×10^{-5} M) and TPA (10^{-7} M) at an 8-d-old mouse myotube. Notice the decline of ACh sensitivity only when the cell was treated with Ptd-Ser (100 μm/ml), and its recovery after washing (*W*). Arrows as in Fig. 1. Resting membrane potential, −50 mV; resting ACh sensitivity, 49 mV/nC.

decrease in its receptor-mediated chemosensitivity. 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 phosphatidylcholine. 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 indispensable 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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