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CYCLIC AMP REGULATES THE LIFE TIME OF ACETYLCHOLINE-ACTIVATED CHANNELS IN CULTURED MYOTUBES

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SUMMARY: 'Giga-seal' patch-clamp recording was performed in embryonic chick myotubes at day 3 to 4 of culture. Myotubes were exposed to agents that enhance the concentration of cytosolic cyclic AMP (cAMPi) and their action on acetylcholine- (ACh) activated channels was investigated. While the conductance and the closed time was unaffected by forsokolin, cholera toxin, dibutyryl cyclic AMP and 8-bromo-cyclic AMP, these agents lengthened the ACh-activated channel life time with efficacy that paralleled with their capability to increase the cAMPi. © 1986 Academic Press, Inc.

The action of several transmitters on the cell membrane is coupled to the activation of a membrane-bound adenylate cyclase (1). This results in an increase of the synthesis of cyclic AMP (cAMP) leading to a change in the ionic conductance that occurs in parallel with protein phosphorylation. The nicotinic acetylcholine receptor (AChR) appears to be associated with the cAMP-dependent protein phosphorylation system since it is rapidly and specifically phosphorylated on its γ and δ subunits by cyclic AMP-dependent protein kinase (2,3). However, the functional significance of the cAMP-dependent AChR-phosphorylation is not clear, since the binding of acetylcholine (ACh) to the nicotinic AChR leads directly to the opening of ion channels (4). It has recently been suggested that this phosphorylation system, in addition to C-kinase system, may regulate the AChR desensitization (5-7).

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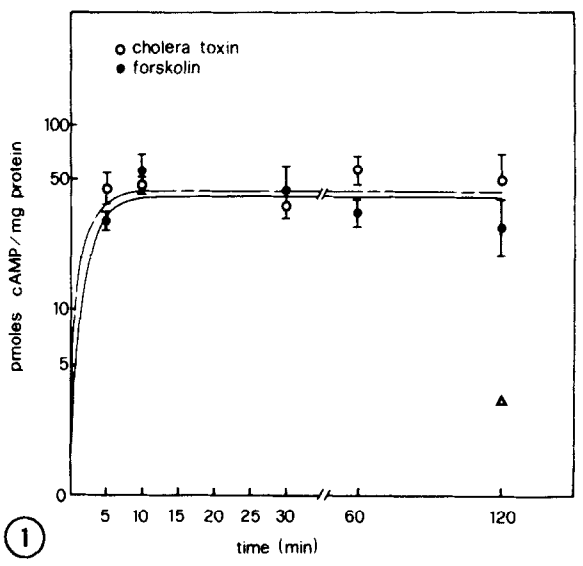
In the present work high resolution ('giga-seal') patch-clamp recording in chick myotubes was used to measure single channel currents from ACh-activated receptors in the presence of increased cytosolic cAMP. We show here that the life time of ACh-activated channels is regulated by cAMP.

EXPERIMENTAL PROCEDURE

Assay of cAMP production. Chick embryo myogenic cells were prepared and cultured as described (8). On the 4th day of culture, cells were incubated in medium containing 1% serum. 95% ethanol plus 0.1% trichloroacetic acid was added to the culture at the end of the incubation with forskolin, dbcAMP, 8-Br-cAMP, cholera toxin or ACh. Doses used were selected for their maximal efficacy in other systems (9). Cells were scraped from the plate and the cell suspension was centrifuged for 30 min at 3000 rpm at 4°C. Supernatants were lyophilized and reconstituted with distilled water. cAMP content was measured by radioimmunoassay (RIA) (10) after suitable dilution and acetylation (11). The RIA had a sensitivity of 2-4 x 10^{-15} M cAMP concentration, an intrassay and interassay coefficient of variation of 5% and 10% respectively. Protein content was measured in the precipitated pellets by the method of Lowry (12). Electrophysiology. Experiments were performed at 20-22 °C on single 3-6 day old multinucleate myotubes equilibrated in minimum essential medium (MEM) buffered with 10 mM NaOH-Hepes at pH 7.2. Membrane currents were recorded via the patch-pipette (13) in cell-attached recording configuration. The pipette contained the standard external solution plus 1 x 10^{-7} M ACh. This ACh concentration was chosen to give a convenient rate of channel openings, without causing appreciable desensitization. In order to examine with accuracy the mean channel open time, patches without double openings were considered. During application of negative pressure, seal resistance between 20 and 60 GΩ generally formed on myotubes. Records of currents were stored on an FM tape recorder, subsequently low-pass filtered at 2 kHz (Kemo filter, type 2BF8), sampled and stored digitally on a computer (IBM PC XT). The sampling interval was 0.122 ms. Individual open times were measured and stored to construct frequency-of-occurrence histograms. Between 300 and 1000 events from each patch were used to construct open-duration and amplitude histograms at each potential. Single channel recordings were analyzed by ignoring all channel closures shorter than 0.1 ms. In all experiments the membrane potential was determined before patch-clamp recordings by using an intracellular electrode, 40 to 60 MΩ resistance, filled with 3 M KCl. The substances were added to the extracellular fluid (2 ml volume) from stock solutions of forskolin (10^{-2} M), dibutyryl cAMP (dbcAMP; 10^{-2} M), 8-bromo-cAMP (8-Br-cAMP; 10^{-2} M) and cholera toxin (1 mg/ml).

RESULTS

Intracellular cAMP assay. Forskolin stimulates the ubiquitous enzyme adenylate cyclase in its catalytic unit leading to a relevant increase intracellular cAMP level (cAMPi) (14). When myotubes were exposed to forskolin ($1x10^{-5}$ M), cAMPi increased to about fourteen-fold within 15-30 min. Cholera toxin (1 μg/ml), that is known to stimulate the N_s unit of the



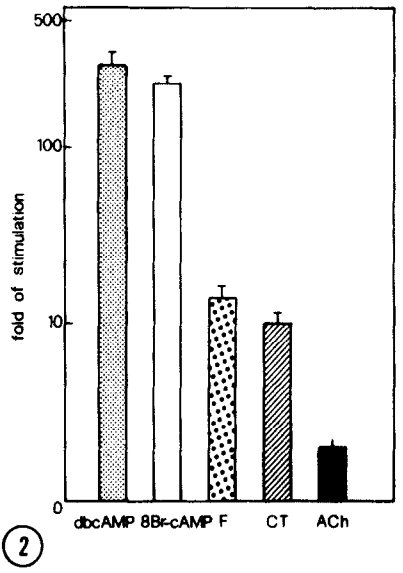


Fig. 1. Time course of intracellular cAMP production following stimulation with forskolin or cholera toxin.
On the fourth day of culture cells were incubated for 2 h in a medium supplemented with 1% serum. The intracellular production of cAMP was measured by RIA (see methods) at various time periods following addition of either forskolin (10^{-5} M) or cholera toxin (1μg/ml). Each point represents mean±S.D. of three different plates, each assayed in duplicate. The open triangle indicates the control value.

Fig. 2. Histogram of n-fold increase of intracellular cAMP following 30 min-stimulation of myotubes with various substances.
Drugs were added to the medium at the following concentrations: dcAMP, 10^{-4} M; 8-Br-cAMP, 10^{-4} M; forskolin (F), 10^{-5} M; cholera toxin (CT), 1 μg/ml; ACh, $3x10^{-5}$ M. For more details see legend to Fig.1.

adenylate cyclase system (14), increased cAMPi to approximately the same values (eleven-fold) and with same time course than forskolin (Fig. 1). Both dbcAMP and 8-Br-cAMP (10^{-4} M) that are able to enter cell membrane, rapidly increased cAMPi to more than 500-fold within 5-15 min. When myotubes were exposed to ACh (10^{-5} M) for 5-20 min, cAMPi was significantly increased to a two-fold value. The levels of cAMP remained substantially stable for more than two hours in the presence of all these agents. Fig. 2 provides a summary of the assay of cAMPi in myotubes exposed to the various drugs.

Single-channel recording. Single-channel currents activated by ACh were recorded from chick myotubes by using cell-attached patch-clamp method (13). Recordings of ACh-activated channels at membrane potentials between -20 to -80 mV gave a mean value for the single channel γ of 52 ± 2 pS (mean±s.e.m.; sixteen patches) (e.g. Fig. 3A) based on an equilibrium potential of -3 mV

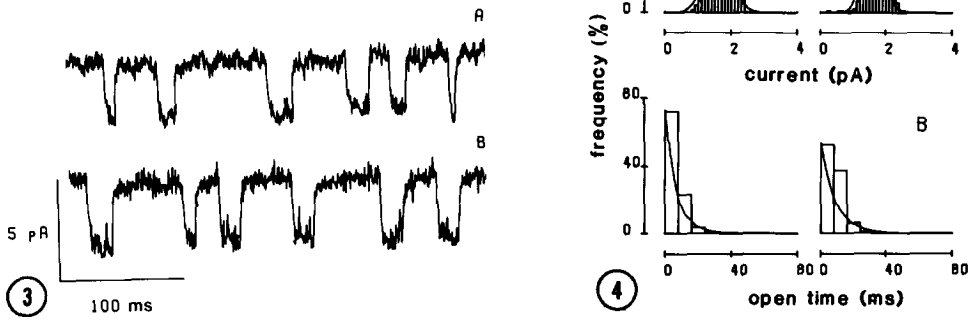


Fig. 3. Samples of single channel currents recorded from 4 day old chick myotubes. The recordings were performed under cell-attached patch configuration. A, standard medium; B, in the presence of forskolin (10^{-5} M; 30 min of exposure). Notice mean open time significantly longer in B than in A. Temperature, 22 °C; holding potential of both myotubes, -50 mV.

Fig. 4. Distribution of amplitude (A) and duration (B) of ACh-activated currents from two different cell-attached membrane-patches in the presence of standard medium (left) and of forskolin-medium (10^{-5} M) after incubation of a myotube for 30 min. Both 4-day myotubes were held at -35 mV membrane potential. Number of events, 267 in the control and 334 in forskolin-medium. (A) mean ACh-activated channel amplitude: 1.68 pA (control, left)); 1.66 pA (forskolin-medium, right). (B) Decay time constant: 6.7 ms (control, left); 8.8 ms (forskolin-medium, right).

(15). This value compares with 54 pS reported for one type of ACh-activated channels on rat myotubes (16). The mean channel open time (τ) was 7.7 $\pm$ 0.4 ms (sixteen patches) at the membrane potential of -30 mV. For each histogram from an experiment τ corresponded to the decay constant of the fitted single exponential (e.g. Fig. 4). The channel closed time of the myotubes examined ranged from 10 to 300 ms.

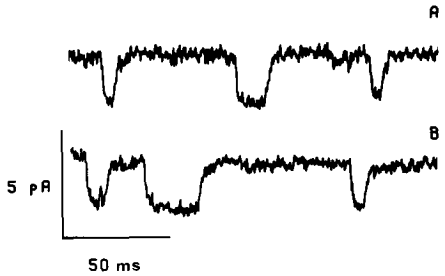


Fig. 5. Samples of ACh-induced openings in 4 day myotubes exposed to the standard medium (A), and to the 8-Br-cAMP- (10^{-4} M) medium (B), under cell-attached mode. Holding potential of both myotubes, -30 mV. Notice the same amplitude and frequency of the openings in both traces, and the lengthening in the open time in B vs A.

Table 1. Functional properties of AChRs in chick cultured myotubes

treatment	γ(pS)	τ(ms)	τ/τ^*
control	52±2 (16)	7.7±0.4	1
forskolin (10^{-5} M)	51±4 (5)	9.3±0.7	1.2
cholera toxin (1μg/ml)	47±3 (3)	10.1±1.2	1.3
dbcAMP (10^{-4} M)	50±2 (7)	10.8±0.8	1.4
8-Br-cAMP (10^{-4} M)	48±4 (3)	14.6±2.5	1.9

Data are means (±s.e.m.) obtained in the time interval of 30 min after drug applications. The values of τ have been determined at membrane potential of -30 mV (resting membrane potential determined intracellularly of all myotubes tested, -32±3 mV). Values in brakets indicate number of myotubes examined under cell-attached mode. τ^* indicates the life time of the control.

Agents which increase cAMPi may affect the ACh-sensitivity of chick myotubes by three different ways: (i) an alteration in single-channel conductance; (ii) an alteration in the kinetics of the channels or (iii) a change in the number of channels capable of being activated. To evaluate these possibilities, we examined the single-channel currents in myotubes exposed to forskolin ($1x10^{-5}$ M). The mean value of γ and of closed time were equivalent to those determined in standard medium, while the mean value of τ significantly differed from the control (e.g. Fig.s 3B and 4). The lengthening of τ following forskolin application is probably related to its action in increasing cAMPi since it was mimiked by cholera toxin (1 μg/ml), and dbcAMP ($1x10^{-4}$ M) with an efficacy that increases with cAMPi enhancement (see Fig. 2 and Tab. 1). Fig. 5 provides an example of the action of 8-Br-cAMP on the ACh-activated openings. The more potent action of 8-Br-cAMP than dbcAMP on the channel life time, could be related to the fact that this analogue of cAMP is poorly hydrolyzed by phosphodiesterases (17). Table 1 provides a summary of the experiments showing that, after 30 min of drug exposure, for all drugs γ was similar while τ was significantly longer compared to control values.

DISCUSSION

Recent studies have provided evidence for a role of protein phosphorylation in the regulation of the functional properties of the nicotinic AChR. C-kinase system phosphorylates α and δ subunits of AChR (3) leading to a change in the gating behaviour of the channel (7,18). cAMP-dependent kinase system phosphorylates γ and δ subunits (2,3) leading to an increase in the

rate of the rapid desensitization of AChR (5,6). In the present work we have investigated whether cAMPi could regulate the nicotinic AChR-channel in an in vitro embryonic muscle fibre system. We show here that agents which cause an increase in cAMPi, elicit also an increase in the mean life time without affecting conductance and closed time of the ACh-activated channel. This lengthening in the channel life time has not been observed by Albuquerque and collegues in adult muscle exposed to forskolin (5). Such a discrepancy could be due to differences in the sytems used (embryonic in this communication vs adult in Albuquerque et al.(5)); or to the way of forskolin application (forskolin applied for a few minutes to the patch-membrane in Albuquerque et al. (5) and for several minutes to whole cells in our experiments). The increase in life time in the presence of the agents examined is related to their efficacy of increasing cAMPi. This indicates that the second messenger cAMP might regulate the gating behaviour of the nicotinic AChR-channel. The lengthening of τ could be due to the phosphorylation of the δ subunit since it has recently been proved that δ subunit modulates the open time of the AChR-channel (19).

In conclusion, the present data indicate that agonists stimulating the adenyl cyclase system may affect the AChR function. Since ACh itself may increase cAMPi (see Fig. 2), an idea worthy of further study is that tne natural transmitter ACh may regulate its own AChR-channel in the muscle fibre through cAMP-dependent kinase system.

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