Acetylcholine and phorbol esters inhibit potassium currents evoked by adenosine and cAMP in *Xenopus* oocytes

(muscarinic response/purinergic response/protein kinase C/second messengers/neurotransmitter interactions)

NATHAN DASCAL*, ILANA LOTAN†, BOAZ GILLO†, HENRY A. LESTER*, AND YORAM LASS†

*Division of Biology, California Institute of Technology, Pasadena, CA 91125; and †Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel

ABSTRACT In *Xenopus laevis* oocytes, adenosine and other purinergic agonists induce a K^+-conductance increase that is fully mimicked by intracellular application of cAMP. Acetylcholine suppresses the K^+-conductance increase caused by adenosine, by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, or by intracellular injection of cAMP. This effect of acetylcholine is not mimicked by intracellular injection of Ca^{2+} or of the Ca-mobilizing agent inositol 1,4,5-trisphosphate. However, adenosine and cAMP responses are inhibited by 4β-phorbol 12,13-dibutyrate and 4β-phorbol 12-myristate 13-acetate. These results suggest that, in *Xenopus* oocytes, the muscarinic inhibition of purinergic and cAMP responses is mediated through the activation of the phospholipid-dependent, Ca-activated protein kinase (protein kinase C).

Several hormones and neurotransmitters produce "slow" membranal responses through changes in the intracellular levels of second messengers, such as cyclic nucleotides, calcium, etc. (for reviews, see refs. 1–4). The best known examples involve activation of adenylate cyclase, followed by elevation of intracellular cAMP concentration, resulting protein phosphorylation, and alterations in the gating of ion channels. This mechanism accounts for the regulation of Ca channels by β-adrenergic agonists in the heart (see refs. 5 and 6 and references therein) and of K^+ channels by neurotransmitters in molluscan neurons (7, 8). Hormones of a second group [e.g., acetylcholine (AcCho) at the muscarinic receptor] are believed to antagonize the effects of adenylate cyclase-activating agents either through inhibition of adenylate cyclase (9, 10) or through activation of a cyclic nucleotide phosphodiesterase (11–13). However, resulting effects on channel gating are not yet fully characterized.

We have chosen the *Xenopus laevis* oocyte as a model system for the study of neurotransmitter interactions of the kind described above. The oocytes exhibit muscarinic (14, 15) and purinergic (16) responses. The dominant response to bath application of adenosine is an increase in K^+ conductance (16) that is (*a*) mediated through P1 purinoreceptor (known to activate adenylate cyclase, ref. 17), (*b*) associated with an increase in cAMP level, (*c*) inhibited by intracellular injection of protein kinase inhibitors, and (*d*) mimicked by intracellular injection of cAMP (36). AcCho, acting on a muscarinic receptor, causes polyphosphoinositide (phosphatidylinositol phosphate) breakdown (18) and also evokes a complex membrane response, consisting of a biphasic Cl^- current, Cl^- current fluctuations, and a less prominent K^+ conductance increase (19). The Cl^- current response apparently results from an inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-mediated increase in the intracellular Ca level (18, 20). The increase may possibly be accompanied by an activation of the Ca/calmodulin-dependent phosphodiester-

ase found in the oocytes (21, 22). In addition, AcCho inhibits adenylate cyclase in the oocyte (23). One may expect, therefore, that AcCho will antagonize the adenosine response.

We report here that AcCho does, indeed, inhibit the K^+-conductance increase evoked by adenosine. We also present evidence that, surprisingly, this effect does not seem to be mediated either by adenylate cyclase inhibition or by the activation of a cyclic nucleotide phosphodiesterase; rather, activation of protein kinase C appears to be involved.

MATERIALS AND METHODS

Ovarian lobes were removed from adult female *Xenopus*. Fully grown, stage 5 and 6 (24) oocytes were separated manually and used as follicles; i.e., the surrounding cellular and noncellular layers were not removed. Both adenosine and AcCho responses in the oocyte originate at the oocyte membrane (14–16). A single cell was placed in a 1-ml bath, constantly perfused with either Ringer's or ND96 solution (pH 7.5, room temperature). The solutions had the following compositions: Ringer's, 116 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Tris/HCl; ND96, 96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes/NaOH. Bath applications of all substances were made without changing the rate of flow; perfusion "dead time" was 3–6 sec. 4β-Phorbol 12,13-dibutyrate (PBt₂) and 4β-phorbol 12-myristate 13-acetate (PMA) were dissolved in dimethyl sulfoxide at 1 mM and stored at $-20°C$. Dimethyl sulfoxide did not have any effects on the oocytes' membrane conductance at concentrations up to 0.3%. Final concentrations of the phorbol esters were prepared just prior to each experiment. All substances used in the study were purchased from Sigma; Ins(1,4,5)P_3 was generously provided by Y. Oron (School of Medicine, Tel Aviv University, Israel).

The electrophysiological procedure has been described in detail (19). Briefly, the cell was penetrated with two conventional 3 M KCl electrodes, voltage-clamped at -50 or -60 mV, and AcCho was applied. The Cl^- equilibrium potential ($V_{Cl,eq}$) was then measured as the reversal potential of the fast or the fluctuational phases of the AcCho response, both purely Cl^- currents (19, 25). The reversal potential of the fluctuational response was determined by manually changing the holding potential during a long-lasting application of AcCho, until the potential at which the fluctuations nullified was found. The reversal potential of the fast Cl^- current was determined by applying a voltage ramp to the cell; the voltage and current outputs of the clamp circuit were connected to the X- and Y-axis inputs, respectively, of a pen recorder. The

Abbreviations: AcCho, acetylcholine; *i*BuMeXan, 3-isobutyl-1-methylxanthine; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PBt₂, 4β-phorbol 12,13-dibutyrate; PMA, 4β-phorbol 12-myristate 13-acetate; $V_{Cl,eq}$, Cl^- equilibrium potential.

reversal potential of the response was at the intersection of the voltage–current curves obtained at rest and at the plateau of the response. Membrane conductance was determined from the slope of the voltage–current curves at −50 mV (close to the resting potential value). In the intervals between drug applications, the voltage-clamp circuit was usually disconnected, so that no current was applied. A few minutes before and during the application of the substances under study, the circuit was reconnected. The intervals between the subsequent applications of AcCho or adenosine were about 30 min, to avoid the effects of agonist-induced self-refractoriness (16, 19).

Intracellular pressure injections of $CaCl_2$ and of $Ins(1,4,5)$-P_3 were made through an additional micropipette, as described (18–20). The injecting pipettes were filled with 10–50 mM $CaCl_2$ (pH 7) or with 1 mM $Ins(1,4,5)P_3$ (pH 7). The amount of injected substance was determined from the volume of the drop released by the pipette into paraffin oil (tested before insertion of the pipette into the cell and, in most cases, after withdrawal). The volume of the injected solution did not exceed 0.5% of the oocyte volume, and the injection artifacts were small or negligible (19). cAMP was injected iontophoretically through a third micropipette containing 0.2 M cAMP (Na salt; pH 7). Injected current was measured by a virtual-ground amplifier.

RESULTS

First, we tested the prediction that AcCho should antagonize adenosine-evoked K^+ current. To minimize the interference by the Cl^- currents, the experiments were performed at membrane potentials near $V_{Cl,eq}$ (unless stated otherwise). The latter was usually between −20 and −30 mV (19); it was estimated either in the oocyte under study or in another oocyte of the same frog (values among cells from the same donor agreed within ±2 mV; ref. 19). Under these conditions,
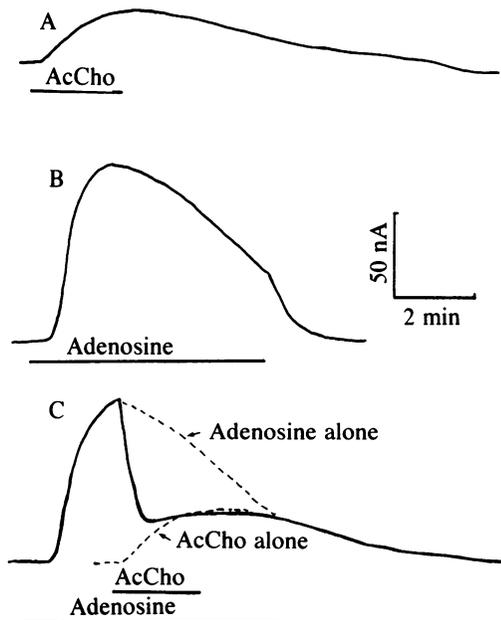

FIG. 1. The effect of 1 μM AcCho on the oocyte's response to 10 μM adenosine. The oocyte was voltage-clamped at −25 mV (equal to $V_{Cl,eq}$ in this cell). The intervals between the applications shown in A, B, and C were 35 min. (A) Response of the oocyte to AcCho alone. (B) Response to adenosine alone. (C) Effect of AcCho, applied at the plateau of the adenosine response. To emphasize the AcCho effect, the records in A and B were superimposed on that in C (dashed lines; adenosine response was corrected for the slight decrease in amplitude in C as compared to B).

AcCho evoked only or predominantly K^+ current (e.g., Fig. 1A).

Bath application of AcCho (1–2 μM) rapidly suppressed the K^+ current evoked by continuous exposure to adenosine (Fig. 1). The residual outward current was usually comparable to that evoked by AcCho alone (Fig. 1C). In all cases, the amplitude of the adenosine response returned to the control level 20–30 min after AcCho washout. The effect of AcCho was blocked by 1 μM atropine (three cells).

The inhibition by AcCho of the adenosine-evoked K^+ conductance increase was studied further in oocytes in which AcCho alone caused a relatively small conductance increase (Fig. 2). Here, at a holding potential of −92 mV, AcCho evoked a net inward current (Fig. 2A); the reversal potential of this response, as measured by the ramp method (Fig. 2C), was −53 mV, indicating an increase of both Cl^- and K^+ conductances (19). During this response, the oocyte's membrane conductance increased by 50% (from 1.6 μS at rest to 2.4 μS at the plateau of the response). Application of adenosine (30 min later) resulted in an outward current (Fig. 2B), accompanied by a 300% conductance increase as compared to rest (6.4 μS at the plateau of the adenosine response; Fig. 2D). When AcCho was applied in the continuous presence of adenosine, the outward current was rapidly transformed into an inward one that had an absolute amplitude (as measured from the resting level of the holding current) comparable to that seen with AcCho alone (Fig. 2B). This was also accompanied by a decrease of membrane conductance to the level seen previously with AcCho alone (2.4 μS; Fig. 2D). Thus, it appears that AcCho indeed blocks the K^+ conductance opened by adenosine; in contrast, adenosine has no obvious effect on AcCho response.
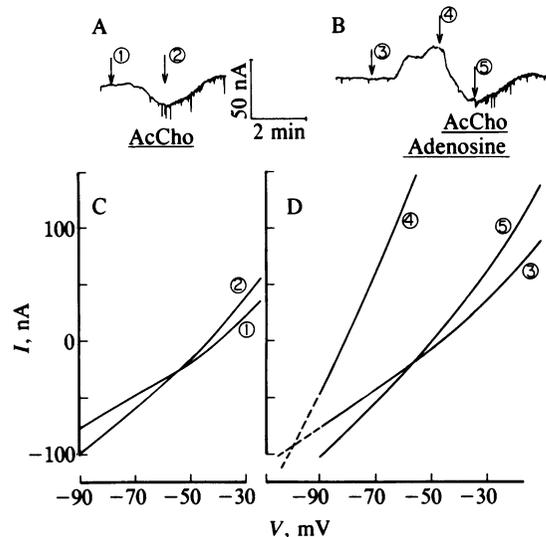

FIG. 2. The inhibition by AcCho of the adenosine-evoked conductance increase. (A) Response of the oocyte to 1 μM AcCho. Holding potential was −92 mV. Numbered arrows indicate the application of voltage ramps at rest (arrow 1) and at the plateau of AcCho response (arrow 2). (B) Responses of the same cell 35 min later. Holding potential as in A. Voltage ramps were applied at rest (arrow 3), at the plateau of the adenosine (10 μM) response (arrow 4), and at the peak of the AcCho response (arrow 5). Between the recordings represented in A and B, the cell was kept at its resting potential (−48 mV). (C) Voltage–current curves during the application of the ramps 1 and 2 shown in A (see *Materials and Methods*). (D) Voltage–current curves corresponding to ramps 3–5 shown in B. Note that extrapolation (broken lines) of the "resting" curve (curve 3) and the curve obtained at the plateau of adenosine response (curve 4) gives an estimate of about −100 mV for the reversal potential of adenosine response; this is close to the K^+ equilibrium potential in these cells (16).

Because the adenosine response in the oocyte appears to be mediated by the activation of adenylate cyclase and elevation of cAMP level, it seemed probable that AcCho exerts its inhibitory effect through the inhibition of adenylate cyclase. Therefore, we tested the effect of AcCho on K^+ current evoked by direct intracellular iontophoretic injection of cAMP (26), thus bypassing the activation of adenylate cyclase. Application of 1–2 μM AcCho at the plateau of cAMP response suppressed the cAMP-induced current by 95 ± 14% (mean ± SD; $n = 10$), leaving a residual outward current that, in most cases, approximately equaled the current produced by AcCho alone (Fig. 3A). This implies that AcCho blocks the adenosine response at a stage later than the activation of adenylate cyclase.

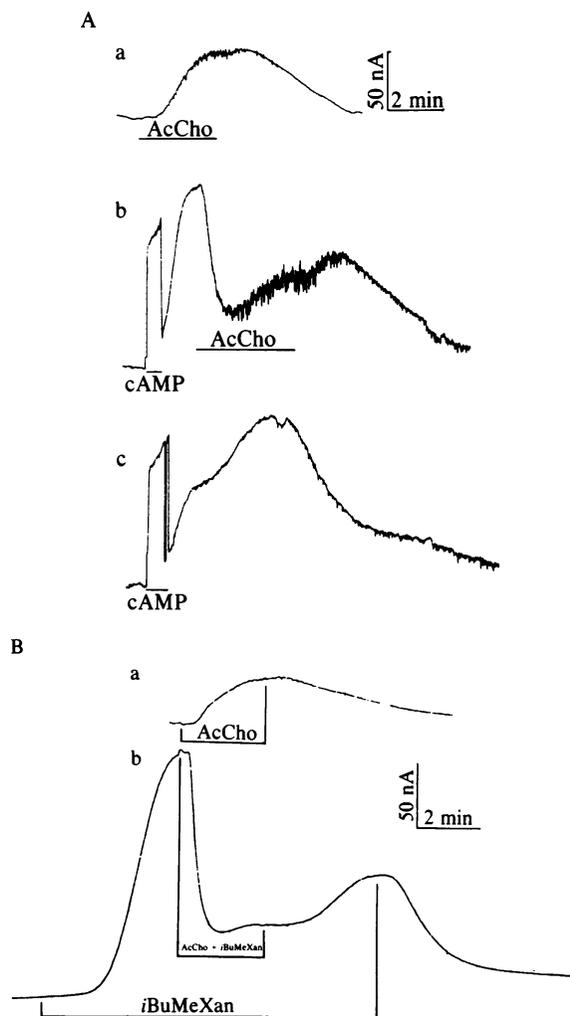AcCho elevates intracellular Ca and cGMP levels in

various tissues (27) and may thus accelerate cAMP hydrolysis by activating either Ca- or cGMP-dependent phosphodiesterases. The effect of 3-isobutyl-1-methylxanthine (*i*BuMeXan), a compound known to inhibit both phosphodiesterases (28), was therefore tested. *i*BuMeXan (0.3–1 mM) significantly enhanced the cAMP response (26, 36) but did not impair the ability of 1 μM AcCho to inhibit cAMP response completely (six cells). Moreover, in some oocytes, 1 mM *i*BuMeXan alone evoked a K^+ current, presumably due to elevation of the cAMP level; in such cells, AcCho completely abolished the *i*BuMeXan-induced outward current (Fig. 3B). Thus, it appears that the inhibitory effect of AcCho is not mediated by activation of a phosphodiesterase.

Injections of up to 200 pmol of $CaCl_2$ per oocyte, which at holding potentials of −40 to −50 mV produced Cl^- currents of 50–150 nA, had no effect on the adenosine response (eight cells; Fig. 4A). Basically negative results were also obtained with intracellular injections of 0.5–3 pmol of Ins(1,4,5)P$_3$: a partial inhibition (Fig. 4B) was observed in two cells, and no effect (as in Fig. 4C) was observed in six oocytes.

The possibility that activation of protein kinase C is involved in the inhibitory action of AcCho on adenosine and cAMP responses was tested by use of the phorbol esters PBt$_2$ and PMA. At concentrations up to 3 μM, neither of the phorbol esters elicited Cl^- or any other current at any holding potential in the range tested (−20 to −80 mV). Therefore, in these experiments the cells were not clamped at $V_{Cl,eq}$.

Application of 1 μM PBt$_2$ during the plateau of either adenosine or cAMP response rapidly and completely suppressed the K^+ current (Fig. 5A). A similar, but slower or partial, effect of PBt$_2$ on cAMP response was observed with 0.1 μM PBt$_2$ [Fig. 5B, trace b; 78.6 ± 22.5% inhibition (SD, $n = 5$)]. With this concentration of PBt$_2$, an apparent partial recovery of cAMP response (after the initial drop) was observed in some cells; this effect was not studied further. PMA (up to 1 μM) usually had no obvious immediate effect when applied at the plateau of adenosine response but inhibited the response effectively when applied several minutes before adenosine (see below).
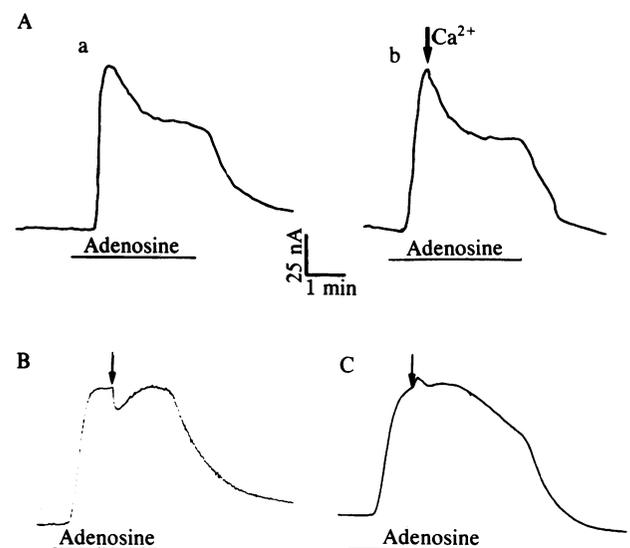

FIG. 3. Inhibition by AcCho of the response of an oocyte to intracellular injection of cAMP (A) and to bath application of *i*BuMeXan (B). (A) Holding potential was −33 mV, about 2 mV more negative then $V_{Cl,eq}$ in this cell. Recording a: the response to 2 μM AcCho. Recording b: 35 min later; AcCho was applied at the plateau of the response to iontophoretic injection of cAMP (30 sec, 105 nA). Recording c: response of the same oocyte to an additional cAMP injection (40 sec, 105 nA), 35 min after the beginning of AcCho washout in recording b. The longer cAMP injection was necessary because of the long-lasting (up to several hours) "refractory period" that follows the first cAMP injection, during which the response to cAMP or adenosine is attenuated (36). (B) Responses of a different oocyte; holding potential was −21 mV (equal to $V_{Cl,eq}$ in this cell). Recording a: Response to 1 μM AcCho alone. Recording b: AcCho (in Ringer's solution containing 1 mM *i*BuMeXan) was added at the plateau of the response evoked by 1 mM *i*BuMeXan.

FIG. 4. The effects of intracellular pressure injections of Ca^{2+} and Ins(1,4,5)P$_3$ on the adenosine response. (A) Holding potential was −24 mV (equal to $V_{Cl,eq}$ in this cell); adenosine concentration was 50 μM. Recording a: control response to adenosine. Recording b: effect of injection of 150 pmol of $CaCl_2$. (B and C) Lack of effect of injection of Ins(1,4,5)P$_3$ (0.5 pmol in B, 2.5 pmol in C) on adenosine (20 μM) response in two different oocytes of the same frog. Holding potential was −20 mV in both cases.
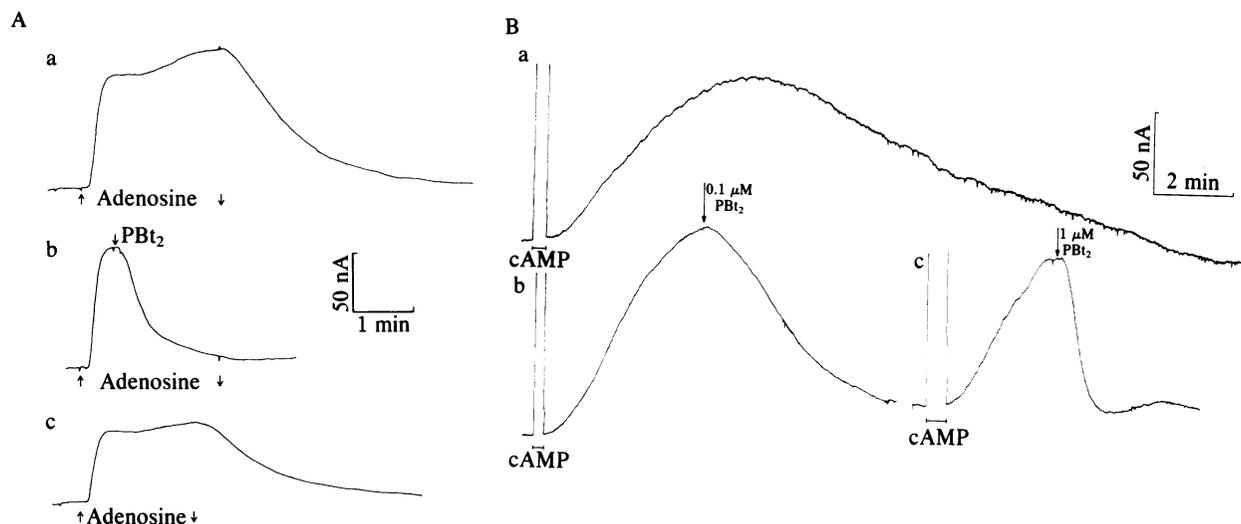
FIG. 5.   Effects of PBt$_2$ on adenosine (*A*) and cAMP (*B*) responses. (*A*) Holding potential was −35 mV. Recording a: the control response to 100 μM adenosine. The reproducibility of the adenosine response was verified by two additional adenosine applications, at 30-min intervals. Recording b: PBt$_2$ was applied after the third adenosine application, at the plateau of the adenosine response. Recording c: after a 60-min washout, the adenosine response was still smaller than before the application of PBt$_2$. (*B*) The control response to iontophoretic cAMP injection (recording a) and the effect of 0.1 μM (recording b) and of 1 μM (recording c) PBt$_2$ on the cAMP response in three different oocytes of the same frog. Holding potentials were −40 mV in a, −35 mV in b, and −45 mV in c. Injection current was 180 nA in all cases; injection times were 20 sec in a, 15 sec in b, and 30 sec in c.

Pretreatment of the oocyte for 2–4 min with 20 nM PBt$_2$ caused a 52 ± 16% (SD, $n = 3$) reduction of the adenosine response; complete inhibition was attained at 0.1 μM. A 15–30 min preincubation with 5 nM PMA resulted in a 73 ± 25% (SD; $n = 3$) inhibition of adenosine response; at 20 nM, the inhibition was 95 ± 8% (SD; $n = 8$). The effect of PMA pretreatment on cAMP response was very similar: a 78 ± 16% (SD; $n = 6$) inhibition at 5 nM, a 87 ± 17% (SD; $n = 8$) inhibition at 10 nM, and complete inhibition at ≥50 nM. The inhibitory effect of PMA could not be reversed by up to 90-min washings.

DISCUSSION

The mechanism of interactions among hormones and neurotransmitters acting through second messengers, at the level of their membrane effects, is not sufficiently explored, although the importance of such effects can hardly be questioned. A comprehensive investigation of such interactions requires a preparation that fulfills the following criteria: (*a*) the cell's membrane should exhibit well-defined electrophysiological responses to the substances under study; (*b*) the second messenger systems involved in mediation of these responses should be (at least preliminarily) identified; (*c*) the preparation should lend itself to rigorous electrophysiological recordings and to manipulations of the intracellular content of the putative second messengers; (*d*) the preparation should provide a homogeneous tissue for a biochemical study. *X. laevis* oocytes appear to fulfill all these criteria (see the Introduction). Although the biological role of the membrane responses to purinergic and cholinergic muscarinic agonists in this preparation is not known, it seems to be a good model system for the study of the mechanisms of neurotransmitter interactions.

In the present study, we showed that AcCho rapidly and reversibly blocks the increase in K$^+$ conductance evoked either by the purinergic agonist adenosine or by its putative second messenger cAMP. The effect of AcCho was blocked by atropine, suggesting mediation by a muscarinic receptor. A reverse interaction does not take place; i.e., adenosine does not inhibit AcCho-evoked Cl$^-$ current (the main muscarinic response in these cells); on the contrary, cAMP

even appears to enhance the second, slow phase of this response (unpublished observations). Because AcCho is able to inhibit the cAMP response, we conclude that the inhibition of adenylate cyclase plays no significant role in the AcCho–adenosine interaction. This is rather unexpected, as inhibition of adenylate cyclase by AcCho is believed to be one of the two mechanisms by which AcCho antagonizes the effects of adenylate cyclase-activating hormones on cAMP levels (and, presumably, on the cell membrane) (9, 10). Another mechanism of antagonism between AcCho and cAMP-level-elevating hormones, the activation of a cyclic-nucleotide phosphodiesterase (13), also appears not to be involved, because AcCho still suppressed the cAMP response in the presence of the potent nonspecific phosphodiesterase inhibitor *i*BuMeXan. Moreover, in some oocytes *i*BuMeXan elicits an outward current, which probably results from cAMP-level elevation due to phosphodiesterase inhibition; yet AcCho inhibits this current, too.

The enhanced breakdown of phosphoinositides, associated with the action of AcCho and other Ca-mobilizing agents, results in the production of two putative second messengers, Ins(1,4,5)P$_3$ (the Ca-mobilizing agent) and diacylglycerol, which probably acts synergistically with the increased intracellular Ca to activate protein kinase C (3, 29). As mentioned above, there is evidence that this mechanism also operates in *Xenopus* oocytes (18, 20). The increase in cytoplasmatic concentration of free Ca alone cannot account for the effect of AcCho, because intracellularly injected Ca^{2+} and Ins(1,4,5)P$_3$ fail to mimic the AcCho effect fully. On the other hand, PBt$_2$ and PMA inhibit the K$^+$ current elicited by adenosine or by cAMP. PBt$_2$ and PMA are potent protein kinase C activators (29), even in the absence of an increased Ca concentration (30). Other recent studies show that phorbol esters and protein kinase C have profound effects on membrane transport mechanisms (31) and on voltage-dependent Ca (32, 33) and K (34) channels. In our experiments, both phorbol esters were effective at 5–20 nM, concentrations close to those appropriate for specific activation of protein kinase C (29). These results strongly suggest that protein kinase C mediates the AcCho antagonism of the purinergic response.

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It is conceivable that protein kinase C acts through phosphorylation of a specific protein (29, 35). It is not clear at present whether this protein itself is a K^+ channel or one of the proteins in the (unknown) chain of reactions that result in the opening of this channel by cAMP. It is also important to clarify whether this mechanism of neurotransmitter interaction is universal and to what extent it might account for the well-known inhibitory action of Ca-mobilizing agents on hormones and transmitters that elevate cAMP levels.

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