J. Physiol. (1987), **394**, *pp.* 239–248
With 4 text-figures
Printed in Great Britain

PROTEIN KINASE C ACTIVATORS INHIBIT THE INOSITOL TRISPHOSPHATE-MEDIATED MUSCARINIC CURRENT RESPONSES IN RAT LACRIMAL CELLS

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(*Received 26 February 1987*)

SUMMARY

1. Pre-incubation (1–5 min) with 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 8–16 nM), a tumour-promoting phorbol ester known to activate protein kinase C, was found to inhibit acetylcholine-induced Ca^{2+}-dependent K^+ and Cl^- currents in cells isolated from rat lacrimal glands.

2. Previous work showed that the same currents may be elicited by dialysing cells with a high-Ca^{2+} (1·0 μM) solution, with inositol trisphosphate ($InsP_3$), or with guanosine 5′-[γ-thio]triphosphate (GTP-γ-S).

3. After TPA incubation, both types of currents could be elicited by dialysis with elevated Ca^{2+} solutions, although the magnitude of the K^+ current was slightly reduced in comparison with control cells.

4. Responses to intracellular dialysis with $InsP_3$ (20 μM) were similar to those in untreated cells, indicating that the Ca^{2+} release process was unaffected.

5. However, the response to GTP-γ-S (0·2–0·5 mM) dialysis was strongly inhibited in TPA-treated cells.

6. These results suggest that protein kinase C exerts an inhibitory action on the pathway leading from receptor activation to inositol trisphosphate production.

INTRODUCTION

In a variety of cell types, muscarinic agonists induce the production of 1,4,5-inositol trisphosphate ($InsP_3$) by activation of a specific phosphodiesterase, phospholipase C. $InsP_3$ releases Ca^{2+} from stores located in the endoplasmic reticulum (reviewed by Berridge & Irvine, 1984). The ensuing rise in intracellular Ca^{2+} concentration is a major factor governing the cellular response. In exocrine glands, for instance, this Ca^{2+} rise is the direct trigger of protein secretion by exocytosis and of fluid secretion. In view of the importance and generality of these processes, the regulation of the various steps which take place between receptor activation and Ca^{2+} release is attracting widespread attention. It was recently shown using patch-clamp techniques that the Ca^{2+}-dependent channels which are present in the plasma membrane of exocrine cells (Petersen & Maruyama, 1984; Marty, Tan & Trautmann, 1984) may be stimulated by dialysing the cells with $InsP_3$ or with

guanosine 5′-[γ-thio]triphosphate (GTP-γ-S), a non-hydrolysable analogue of GTP (Evans & Marty, 1986*a*). These observations give support to the hypothesis (Birdsall, Hulme & Stockton, 1984) that a GTP-binding protein plays a role in the muscarinic response, most probably as a link between receptor occupation and phospholipase C activation.

In earlier experiments, it was noted that responses to either acetylcholine or GTP-γ-S, but not to $InsP_3$, displayed slow-wave patterns, and it was therefore suggested that feed-back mechanisms were controlling $InsP_3$ production (Evans & Marty, 1986*a*). One possible regulator of the system may be diacylglycerol which is produced, together with $InsP_3$, by the phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate. Diacylglycerol is known to activate protein kinase C, an enzyme which plays a modulatory role in a large variety of cellular processes such as mitosis and secretion (reviewed by Nishizuka, 1984). Concerning muscarinic responses, a recent study (Vincentini, Di Virgilio, Ambrosini, Pozzan & Meldolesi, 1985) has shown that incubation of PC12 cells with phorbol esters inhibits the carbachol-induced phosphoinositide hydrolysis and the subsequent rise in intracellular Ca^{2+}. These results led to the hypothesis that muscarinic receptors are under feed-back control by protein kinase C. In the present work, we describe the effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester known to activate protein kinase C (Nishizuka, 1984) on the responses obtained when isolated cells from rat lacrimal glands are stimulated with acetylcholine, GTP-γ-S, $InsP_3$ and high intracellular Ca^{2+}.

METHODS

The main experimental procedures were as previously described (Marty *et al.* 1984). In short, cells were isolated from rat exorbital lacrimal glands and kept in minimum essential medium (MEM) culture medium for 1–8 h in a 37 °C incubator until use. Tight-seal whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were obtained at room temperature (20–22 °C) after replacement of the incubation medium by a solution containing (in mM): NaCl,140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; HEPES–NaOH, 5; pH 7·2. The standard internal solution contained (in mM): KCl, 140; EGTA–KOH, 0·5; HEPES–KOH, 5; pH 7·2. The EGTA concentration in this solution was chosen to keep the internal Ca^{2+} concentration $[Ca^{2+}]_i$ at a low level in resting cells, while allowing $[Ca^{2+}]_i$ to rise when cells are stimulated with acetylcholine (Marty *et al.* 1984). In some control experiments, the intracellular Ca^{2+} concentration was maintained at a fixed level (1·0 μM) with a high concentration of Ca–HEDTA buffer; the composition of this solution was (in mM): HEDTA–KOH, 40; KCl, 36; $CaCl_2$, 12; HEPES–KOH, 5; pH 7·2. Acetylcholine (ACh) was applied to the bath with a fast solution exchange device (Krishtal & Pidoplichko, 1980) at a concentration of 0·4 μM. This is a concentration at which the ACh-induced increase in Ca^{2+}-dependent currents reaches saturation (Evans & Marty, 1986*a*).

Cells from lacrimal glands possess three types of Ca^{2+}-dependent channels, which are selective for K^+, for Cl^- and for monovalent cations. ACh application activates mostly K^+- and Cl^--selective channels (Marty *et al.* 1984). In most experiments the cells were held at −60 mV, and depolarizing voltage pulses were given to a test potential of 0 mV. This test potential was chosen because it is the Cl^- equilibrium potential, and therefore it allows a good measurement of K^+ currents. The holding potential value was sufficiently hyperpolarized as to render Ca^{2+}-activated K^+ currents small relative to Ca^{2+}-activated Cl^- currents. The holding potential was not set at the K^+ equilibrium potential (−85 mV) because, when cells are held at this value, a progressive increase in the amplitude of Ca^{2+}-dependent currents is often observed, especially in the presence of $InsP_3$ or GTP-γ-S in the internal solution. This evolution is probably due to an influx of Ca^{2+} which accompanies $InsP_3$ production (Llano, Marty & Tanguy, 1987). However, contamination of Cl^-

currents, as measured at −60 mV, by either K^+ currents or cation-selective currents, is likely to be small (at most 20%).

TPA, 1-oleoyl-2-acetyl-glycerol (OAG), and 4α-phorbol 12,13-didecanoate, were purchased from Sigma and kept in ethanol solution stocks. Ethanol doses 100 times higher than those added together with the compounds did not alter muscarinic responses. Incubation of cells with these compounds was done in a 37 °C incubator, by adding them to the Petri dishes in culture medium. Unless otherwise noted, the compounds were washed from the dishes before recording. In order to minimize possible errors due to variations between preparations and evolution of these preparations with time, control and treated dishes were always alternated for recording during a single experimental day.

$InsP_3$ was purchased from Amersham Laboratories; GTP-γ-S was purchased from Sigma.

RESULTS

ACh-induced currents

Stimulation of control cells with 0·4 μM-acetylcholine (ACh) led to the activation of Ca^{2+}-dependent K^+- and Cl^--selective currents, as previously described (Marty *et al.* 1984; Trautmann & Marty, 1984; Evans & Marty, 1986*a*). An example of the responses elicited by ACh is presented in Fig. 1*A*, which shows a continuous recording of the membrane currents obtained as the holding potential was stepped from −60 to 0 mV for 300 ms, at a frequency of 1 Hz. Prior to ACh stimulation, the pulses to 0 mV gave rise to outward currents flowing through Ca^{2+}-dependent K^+ channels (called BK channels). Upon application of ACh (during the time indicated by the bar below the records) the magnitude of the currents recorded during the pulses to 0 mV augmented as the activity of BK channels increased and inward currents developed during the interpulse periods due to the activation of the Ca^{2+}-dependent Cl^- channels. As previously noted (Evans & Marty, 1986*a*), the responses displayed damped oscillations. Using this protocol, K^+-selective currents ($I_{K,0}$) were measured at 0 mV, the Cl^- reversal potential. Currents were also measured at −60 mV, a potential sufficiently close to the K^+ reversal potential (−85 mV) for K^+ currents to be small compared to Cl^- currents ($I_{Cl,-60}$) (see Methods). In control cells, $I_{K,0}$ had magnitudes of 0·19 ± 0·12 nA (mean ± standard deviation; $n = 27$) prior to ACh application. Stimulation with 0·4 μM-ACh led to maximum increases of $I_{K,0}$ to 1·21 ± 0·76 nA and to the activation of $I_{Cl,-60}$ with peak values of −1·08 ± 0·6 nA ($n = 27$).

After treatment with TPA (16 nM for 5 min) most cells failed to respond to ACh (Fig. 1*B*, upper record). Occasionally, small responses were encountered (Fig. 1*B*, lower record). In twenty-four TPA-treated cells studied, baseline K^+ currents at 0 mV ($I_{K,0}$) had slightly smaller amplitudes than those found in control cells (0·13 ± 0·10 nA). In contrast to control cells these currents increased very little upon ACh stimulation (maximum values reached being 0·18 ± 0·20 nA); ACh-activated Cl^--selective currents were almost entirely absent ($I_{Cl,-60}$: −0·03 ± 0·01 nA). These currents were measured 2–30 min after washing out TPA, without any sign of recovery over this time period. The onset of the inhibitory action of TPA seemed rather fast, as two cells which were treated for only 1 min with 16 nM-TPA displayed very weak responses similar to those of Fig. 1*B*. Cells treated with 8 nM-TPA for 5 min gave strongly reduced responses ($n = 2$), but no inhibition was observed after 5 min of incubation with 1·6 nM-TPA ($n = 3$). Incubations with 4α-phorbol 12,13-

didecanoate, a phorbol ester which does not activate protein kinase C (Castagna, Takai, Kaibrichi, Sano, Kikkawa & Nishizuka, 1982), failed to affect the muscarinic response (Fig. 1*C*). In five cells treated with this compound $I_{K,0}$ increased from a baseline level of $0{\cdot}25 \pm 0{\cdot}11$ nA to $2{\cdot}03 \pm 0{\cdot}43$ nA upon ACh application. ACh-activated $I_{Cl,-60}$ had magnitudes of $-1{\cdot}05 \pm 0{\cdot}5$ nA.

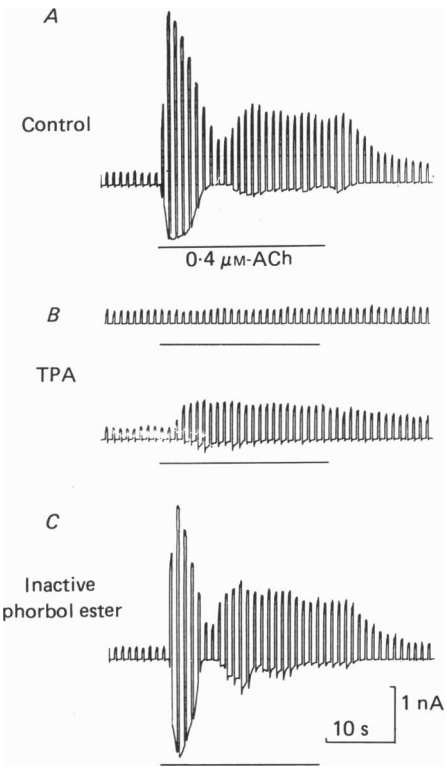

Fig. 1. Effect of TPA on ACh-induced currents. Cell currents were recorded in four different cells from the same preparation. In each experiment voltage pulses to 0 mV were given repetitively from a holding potential of −60 mV. ACh (0·4 μM) was applied externally during the time indicated by the bar below the records. *A*, control cell. *B*, two examples of current responses obtained after 5 min pre-incubation with 16 nM-TPA. *C*, current response in a cell which had been pre-incubated for 5 min with 16 nM-4α-phorbol 12,13-didecanoate.

We have not done an extensive study of the effects of synthetic diacylglycerol analogues in this preparation. However, our experimental data indicates that OAG is able to mimic the effects of TPA, but under different experimental conditions. Thus, pre-incubations with 25 μM-OAG for periods of 20–40 min failed to have any effect on the ACh-induced currents if the compound was washed from the bath before the start of recording as was done in TPA-treated dishes ($n = 6$). Using this experimental protocol, incubations as long as 2 h were required in order to block the muscarinic responses ($n = 3$). However, if, during the time of recording, cells were maintained in an external solution to which 25 μM-OAG was added, relatively short

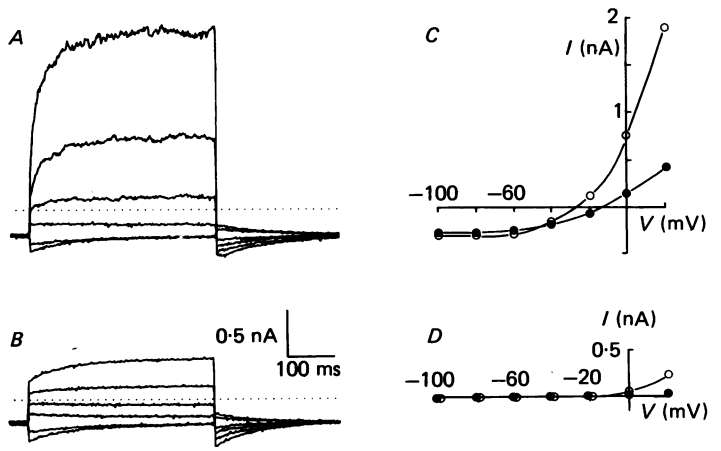

Fig. 2. Ca^{2+}-dependent currents in TPA-treated cells. *A*, *B*, and *C*; cell dialysed with a 1 μM-Ca^{2+} buffer. Holding potential, −60 mV. *A*, responses to voltage steps to −100, −80, −40, −20, 0 and 20 mV in normal saline. *B*, responses to the same voltage steps in the presence of 5 mM-TEA. *C*, *I–V* curves for the records in *A* (○) and in *B* (●). *D*, *I–V* curves from another cell with standard, low $[Ca^{2+}]_i$ internal buffer. ○, normal saline. ●, normal saline with 5 mM-TEA. Both cells were from the same preparation and had been subjected to a 5 min pre-incubation with 16 nM-TPA.

TABLE 1. Ca^{2+}-dependent currents in control dishes and in TPA-treated dishes

TPA treatment	External saline	I_{-60} (pA)	I_{20} (pA)	τ_{20} (ms)
None	Control	−166±144 (8)	1910±1200 (8)	—
	TEA	−155±150 (8)	346±145 (8)	83±18 (4)
5 min pre-incubation	Control	−156±155 (13)	1170±450 (13)	—
	TEA	−134±128 (13)	255±174 (13)	104±28 (5)

Cells were dialysed with a 1 μM $[Ca^{2+}]_i$ solution (see Methods). For each experiment, whole-cell currents were recorded in control external solution and during application of 5 mM-TEA to the bath solution. Cell currents measured at −60 (I_{-60}) or 20 mV (I_{20}) were comparable in control dishes and in dishes which were pre-incubated with TPA (16 nM for 5 min). In certain cells, the time constant of the Cl current relaxations obtained in TEA saline in response to voltage jumps to 20 mV (τ_{20}; holding potential, −60 mV) was determined using a least-square fit routine. Means±S.D., and number of independent measurements (in parentheses) are indicated.

incubation times (20 min) were effective in blocking the ACh responses ($n = 3$). These results may be explained by the more rapid degradation of OAG as compared to TPA and/or by a lower specificity of OAG for the protein kinase C of this preparation.

Ca^{2+}-dependent K^+ and Cl^- currents

In view of the results presented in Fig. 1, it seemed important to test whether Ca^{2+}-dependent K^+ and Cl^- channels were still responsive to rises in $[Ca^{2+}]_i$ after TPA treatment. To address this question cells were dialysed with a solution in which $[Ca^{2+}]_i$ was buffered at 10^{-6} M (see Methods). Voltage pulses were applied from a holding potential of −60 mV to values ranging from −100 to 20 mV. Under these conditions, even after TPA treatment, large voltage-dependent currents were

observed (Fig. 2*A*). In order to separate K^+- and Cl^--selective currents, 5 mM-tetraethylammonium (TEA) was externally applied with the fast bath exchange system. This drug strongly reduces BK currents (Trautmann & Marty, 1984) leaving Ca^{2+}-dependent Cl^- currents unaffected. The currents recorded in the presence of TEA (Fig. 2*B* and *C*) had the same features as the Ca^{2+}-dependent Cl^- current previously described (Evans & Marty, 1986*b*). By comparison, cells dialysed with the standard low $[Ca^{2+}]_i$ solution gave markedly smaller currents (Fig. 2*D*). The results of Fig. 2 thus show that treatment with TPA did not prevent K^+ and Cl^- channels from responding to $[Ca^{2+}]_i$ rises. Table 1 summarizes the results of numerous experiments of the type illustrated in Fig. 2*A–C*, performed in control and TPA-treated cells. As may be seen from this Table, Cl^- currents (as measured in the presence of TEA) have very similar amplitudes and relaxation time constants under both experimental conditions. However, mean currents measured in normal saline at +20 mV are somewhat larger in control cells than in TPA-treated cells. This seemed a consistent result when data were compared in single experimental days, indicating that BK channels lose some of their responsiveness to Ca^{2+} after TPA treatment. A similar effect was recently described in response to large (micromolar) concentrations of phorbol esters for another Ca^{2+}-dependent K^+ conductance in hippocampal pyramidal cells (Malenka, Madison, Andrade & Nicoll, 1986). In any event, the reduction of BK current apparent in Table 1 is far too weak to account for the much more pronounced inhibition of the ACh-induced currents illustrated in Fig. 1. Thus, the main effect of TPA treatment is not due to a direct action on the Ca^{2+}-dependent channels.

$InsP_3$-induced currents

In order to test whether TPA affected the Ca^{2+} release mechanism, responses to intracellular dialysis with $InsP_3$ were compared with and without TPA pre-treatment. An example of the currents obtained from a control cell dialysed with an internal solution supplemented with 20 μM-$InsP_3$ is presented in Fig. 3*A*. The recording starts in the cell-attached mode. Holding potential was set at −60 mV and 300 ms depolarizing pulses to 0 mV were applied at 1 Hz. Shortly after the transition to whole-cell mode (indicated by the arrow) both Ca^{2+}-dependent K^+ and Cl^- channels activated giving rise to outward currents during the depolarizing pulses and inward current tails upon return to the holding potential. Under control conditions (without TPA incubation), the magnitude of the responses to $InsP_3$ dialysis was rather variable, with marked differences between preparations. In various control cells tested, dialysis with 20 μM-$InsP_3$ led, during the first minute of whole-cell recording, to the activation of K^+- and Cl^--selective currents which, measured at 0 and −60 mV, had peak values of $0{\cdot}74 \pm 0{\cdot}51$ and $-0{\cdot}29 \pm 0{\cdot}32$ nA ($n = 9$), respectively. In three cells dialysed with 2 μM-$InsP_3$ these values were $0{\cdot}22 \pm 0{\cdot}07$ (not significantly larger than in the absence of $InsP_3$) and 0 nA.

Pre-incubation with 16 nM-TPA for 5 min did not alter significantly the currents induced by either 2 or 20 μM-$InsP_3$. Figure 3*B* shows a recording from a TPA-treated cell, dialysed with 20 μM-$InsP_3$. The pattern of current activity observed was very similar to that seen in control cells. Thus, $I_{K,0}$ and $I_{Cl,-60}$ were $0{\cdot}50 \pm 0{\cdot}53$ and $-0{\cdot}32 \pm 0{\cdot}33$ nA ($n = 13$) for 20 μM-$InsP_3$-dialysed cells and $0{\cdot}20 \pm 0{\cdot}15$ and 0 nA

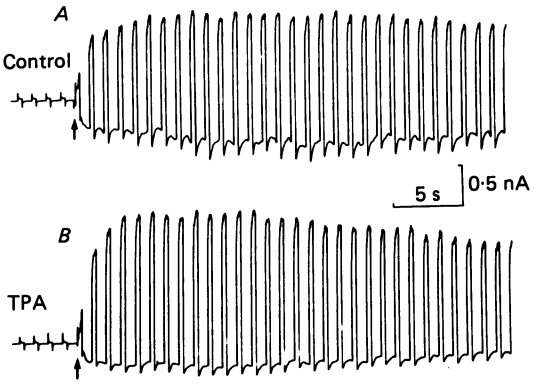

Fig. 3. Lack of effect of TPA on $InsP_3$-induced currents. Recordings from two different cells, from the same preparation, dialysed with 20 μM-$InsP_3$. Each record starts in the cell-attached configuration. Breaking into the cell (start of the whole-cell recording mode) is indicated by arrows. Repetitive pulses to 0 mV were given from a holding potential of −60 mV. *A*, control cell. B, cell which had been exposed to 16 nM-TPA for 5 min.

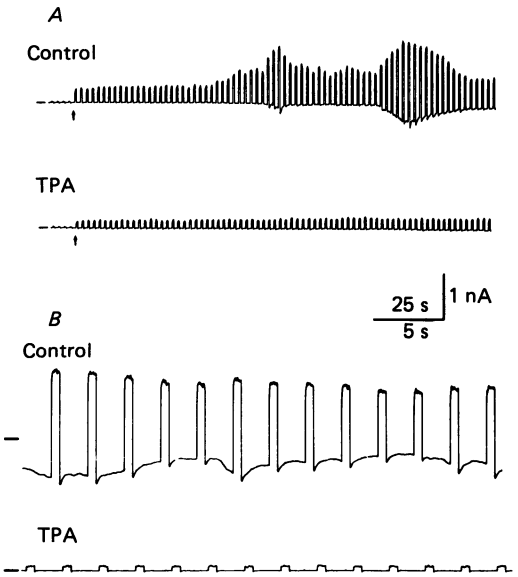

Fig. 4. Effect of TPA on GTP-γ-S-induced currents. *A*, continuous recordings of currents in two GTP-γ-S-dialysed cells, from the same preparation. Recordings start in the cell-attached mode. Transitions to whole-cell are indicated by the arrows. Cell membrane potentials were maintained at −60 mV and stepped to 0 mV with 600 ms pulses. In the control cell (top trace) waves of current activity are induced by GTP-γ-S (0·2 mM). In the cell treated with 16 nM-TPA for 5 min (lower trace) a higher concentration of GTP-γ-S (0·5 mM) failed to elicit any increase in current. Zero current levels are indicated by the bars in front of each record. *B*, currents recorded from two cells dialysed with 0·5 mM-GTP-γ-S. Records were taken 5 min after initiation of whole-cell mode. In the control cell (top trace) sustained activation of currents is observed. This activity is absent in the TPA-treated cell (lower trace). Both cells are from the same preparation. Zero current levels are indicated as above.

($n = 3$) for cells dialysed with 2 μM-$InsP_3$. The lack of difference between the two groups indicates that the action of TPA takes place upstream of the Ca^{2+} release mechanism.

GTP-γ-S-induced currents

A possible site where protein kinase C could exert a regulatory action in the chain of events leading from activation of the muscarinic receptor to production of $InsP_3$ is the GTP-binding protein which presumably mediates the interaction between receptor stimulation and activation of phospholipase C (Cockcroft & Gomperts, 1985; Litosch, Wallis & Fain, 1985). Intracellular dialysis of lacrimal cells with GTP-γ-S, a non-hydrolysable analogue of GTP, activates Ca^{2+}-dependent channels (Evans & Marty, 1986*a*), presumably by stimulating the GTP-binding protein directly. In this section we describe the effects of TPA treatment on the responses to intracellular dialysis with this compound.

The onset of the responses to intracellular dialysis with 0·2 mM-GTP-γ-S in control cells is illustrated in Fig. 4*A* (top). The recording starts in the cell-attached configuration. Approximately 1 min after initiation of cell dialysis, an increase in current magnitude is observed, both during the pulses to 0 mV and during the interpulse periods. This increase in the activity of Ca^{2+}-dependent K^+ and Cl^- channels is repeated in a wave pattern, as described previously (Evans & Marty, 1986*a*). All of ten control cells tested presented this type of current activation, the latency to the appearance of the first wave of current ranging from 30 to 80 s. In TPA-treated dishes (16 nM, for 5 min) the same concentration of GTP-γ-S failed to elicit any increase in current magnitudes during 5 min of recording ($n = 3$). Even higher doses of GTP-γ-S (0·5 mM) were unable to yield the type of current activation observed in control cells (see Figs 4*A* and *B*, lower records). Thus, although at the higher doses of GTP-γ-S control dishes presented a sustained augmentation of Ca^{2+}-dependent currents (Fig. 4*B*, top trace; recording taken after 5 min of initiation of cell dialysis), this effect was absent in cells from TPA-treated dishes. The maximum current values obtained during the first 5 min of whole-cell recording, from control cells dialysed with 0·5 mM-GTP-γ-S, were $-0{\cdot}57 \pm 0{\cdot}44$ nA at -60 mV and $1{\cdot}41 \pm 0{\cdot}87$ nA at 0 mV ($n = 9$). In cells from TPA-treated dishes, the corresponding values were 0 at -60 mV and $0{\cdot}16 \pm 0{\cdot}70$ nA at 0 mV ($n = 8$). These experiments show that TPA treatment strongly inhibits the ability of GTP-γ-S to induce activation of the Ca^{2+}-dependent channels.

DISCUSSION

Incubation with TPA (8–16 nM for 1–5 min) leads to a very marked inhibition of the muscarinic response in isolated rat lacrimal gland cells. This is a concentration range where TPA is thought to act specifically by stimulating protein kinase C (Nishizuka, 1984). A further indication that activation of protein kinase C mediates the TPA effect is provided by the lack of effect of 4α-phorbol 12,13-didecanoate, which is unable to activate the kinase.

Experiments using intracellular dialysis with high Ca^{2+}, $InsP_3$ or GTP-γ-S as stimulators of the Ca^{2+}-dependent channels strongly restrict the number of possible targets of the TPA effect. In cells subjected to a $[Ca^{2+}]_i$ of 1 μM, TPA treatment

slightly reduced the activity of BK channels, while Ca^{2+}-dependent Cl^- currents were similar to those in control cells. A direct effect of protein kinase C activation on BK channels thus seems to be present in this preparation. However, the extent of this inhibition is not sufficient to explain the block of the ACh-induced K^+ response, let alone that of the Cl^- response. Therefore, the inhibition of the muscarinic response by TPA cannot be ascribed to a direct action on Ca^{2+}-dependent channels. The observation that $InsP_3$ retains its ability to activate Ca^{2+}-dependent currents in TPA-treated cells confirms that the Ca^{2+}-dependent channels are not the primary target of TPA action. This finding further shows that the Ca^{2+} release mechanism itself is not affected.

In view of the consistent lack of activation of Ca^{2+}-dependent currents by dialysis with GTP-γ-S in TPA-treated cells, we conclude that the inhibition of the muscarinic response is largely explained by a modification of either the GTP-binding protein, its coupling to phospholipase C, or the activity of the latter. In so far as the observed effects may be considered to be mediated by protein kinase C, these results suggest the possibility that either the GTP-binding protein or phospholipase C may be substrates for phosphorylation by the kinase. Consistent with the former suggestion, it has been found recently that another GTP-binding protein (protein G_i) is phosphorylated by protein kinase C (Katada, Gilman, Watanabe, Bauer & Jakobs, 1985).

Earlier work using measurements of labelled phosphoinositides and/or fluorescent Ca^{2+} indicators led to the suggestion that protein kinase C inhibits receptor-coupled $InsP_3$ production in a variety of systems (reviewed by Moolenar, Defize & De Laat, 1986; Drummond, 1986), including certain muscarinic responses (Vincentini *et al.* 1985; Kanba, Kanba & Richelson, 1986). Our results give new support to this hypothesis. They further show that the inhibitory action of protein kinase C can be demonstrated using single-cell recordings, under conditions which largely preserve complex cellular functions.

Because phospholipase C-produced diacylglycerol is likely to activate protein kinase C, the present findings indicate the existence of negative feed-back control in the pathway of muscarinic stimulation, upstream of the Ca^{2+} release process. This could explain the slow waves observed in both acetylcholine- and GTP-γ-S-induced responses, but not in $InsP_3$-induced responses (Evans & Marty, 1986*a*). It should be borne in mind that such waves also require the presence of a positive feed-back mechanism, the nature of which remains obscure.

It is well documented that, in many preparations, treatment with phorbol esters facilitates exocytosis at a fixed concentration of intracellular Ca^{2+} (reviewed in Nishizuka, 1984). Assuming that this also applies in the present case, our results suggest that, in lacrimal glands, diacylglycerol production would exert both a facilitatory effect (by controlling exocytosis itself) and an inhibitory effect (by reducing the Ca^{2+} concentration) on protein secretion. Fluid secretion, which appears to be linked to the activation of Ca^{2+}-dependent channels (Petersen & Maruyama, 1984; Marty *et al.* 1984), would be only inhibited.

We thank Dr J. C. Louis for helpful discussions. This work was supported by grants from C.N.R.S., D.R.E.T. and Université Pierre et Marie Curie. I. Llano was supported by a C.N.R.S. fellowship.

REFERENCES

BERRIDGE, M. J. & IRVINE, R. F. (1984). Inositoltrisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315–320.

BIRDSALL, N. J. M., HULME, E. C. & STOCKTON, J. M. (1984). Muscarinic receptor heterogeneity. In *Proceedings of the International Symposium on Subtypes of Muscarinic Receptors; Trends in Pharmacological Science, Supplement*, pp. 4–8.

CASTAGNA, M., TAKAI, Y., KAIBRICHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor promoting phorbol esters. *Journal of Biological Chemistry* **257**, 7847–7857.

COCKCROFT, S. & GOMPERTS, B. D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature* **314**, 534–536.

DRUMMOND, A. H. (1986). Inositol lipid metabolism and signal transduction in clonal pituitary cells. *Journal of Experimental Biology* **124**, 337–358.

EVANS, M G. & MARTY, A. (1986*a*). Potentiation of muscarinic and alpha-adrenergic responses by an analogue of guanosine triphosphate. *Proceedings of the National Academy of Sciences of the USA* **83**, 4099–4103.

EVANS, M G. & MARTY, A. (1986*b*). Ca-dependent Cl currents in isolated cells from rat lacrimal glands. *Journal of Physiology* **378**, 437–460.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

KANBA, S., KANBA, K. S. & RICHELSON, E. (1986). The protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inhibits muscarinic (M_1) receptor-mediated inositol phosphate release and cyclic GMP formation in murine neuroblastoma cells (clone N1E-115). *European Journal of Pharmacology* **125**, 155–156.

KATADA, T., GILMAN, A., WATANABE, Y., BAUER, S. & JAKOBS, K. H. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *European Journal of Biochemistry* **151**, 431-437.

KRISHTAL, O. A. & PIDOPLICHKO, V. I. (1980). A receptor for protons in the nerve cell membrane. *Neuroscience* **5**, 2325–2327.

LITOSCH, I., WALLIS, C. & FAIN, J. N. (1985). 5-Hydroxytryptamine stimulates inositol phosphate production in a cell-free system from blowfly salivary glands. *Journal of Biological Chemistry* **260**, 5464–5471.

LLANO, I., MARTY, A. & TANGUY, J. (1987). Dependence of intracellular effects of GTPγS and inositoltrisphosphate on cell-membrane potential and on external Ca ions. *Pflügers Archiv* **409**, 499–506.

MALENKA, R. C., MADISON, D. V., ANDRADE, R. & NICOLL, R. A. (1986). Phorbol esters mimic some cholinergic actions in hippocampal pyramidal neurones. *Journal of Neuroscience* **6**, 475–480.

MARTY, A., TAN, Y. P. & TRAUTMANN, A. (1984). Three types of calcium-dependent channel in rat lacrimal glands. *Journal of Physiology* **357**, 293–325.

MOOLENAR, W. H., DEFIZE, L. H. K. & DE LAAT, S. W. (1986). Tonic signalling by growth factor receptors. *Journal of Experimental Biology* **124**, 359–373.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308**, 693–698.

PETERSEN, O. H. & MARUYAMA, Y. (1984). Calcium-activated potassium channels and their role in secretion. *Nature* **307**, 693–696.

TRAUTMANN, A. & MARTY, A. (1984). Activation of Ca-dependent K channels by carbamylcholine in rat lacrimal glands. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 611–615.

VINCENTINI, L. M., DI VIRGILIO, F., AMBROSINI, A., POZZAN, T. & MELDOLESI, J. (1985). Tumor promoter 12-myristate, 13-acetate inhibits phosphoinositide hydrolysis and cytosolic Ca^{2+} rise induced by the activation of muscarinic receptors in PC12 cells. *Biochemical and Biophysical Research Communciations* **127**, 310–317.