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The effect of a phorbol ester upon the cholinergic regulation of potassium permeability in the rat submandibular gland

J. D. Pediani and S. M. Wilson

The Institute of Physiology, University of Glasgow, Glasgow G12 8QQ (Scotland, UK)
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Abstract. Acetylcholine releases calcium from cytoplasmic stores and permits an influx of calcium in salivary acinar cells. The resultant rise in $[Ca^{2+}]_i$ causes an increase in potassium permeability which is an important part of the secretory response. We have investigated the effects of 12-0-tetradecanoyl phorbol-13-acetate, a potent activator of protein kinase C, upon this regulation of potassium permeability in superfused pieces of rat submandibular salivary gland. This compound inhibited the initial $[Ca^{2+}]_0$-independent component of the response of acetylcholine but had no effect upon the subsequent $[Ca^{2+}]_0$-dependent phase. This compound does not, therefore, appear to inhibit receptor-regulated calcium influx.
Key words. Salivary gland; phorbol ester; potassium permeability; $^{86}Rb^+$-efflux; stimulus-response coupling; protein kinase C.

Exocrine fluid secretion is regulated, primarily, via muscarinic receptors that are functionally coupled to phosphoinositidase C. Occupation of these receptors thus allows acetylcholine to regulate the rate of hydrolysis of phosphatidylinositol bisphosphate (PIP_2), a membrane phospholipid. The hydrophilic product of this reaction, inositol-1, 4, 5-trisphosphate (IP_3), enters the cytoplasm and releases calcium from a cytoplasmic store[1]. The resultant rise in internal free calcium concentration ($[Ca^{2+}]_i$), which is subsequently sustained by a regulated influx of calcium, evokes the co-ordinated increase in the chloride and potassium permeability of the plasma membrane that underlie secretion[2].

The hydrophobic product of PIP_2 hydrolysis, a diacylglycerol molecule, is also biologically active. This compound remains in the membrane and allosterically modulates the activity of protein kinase C, a calcium and phospholipid-dependent enzyme that exerts control over several aspects of cellular metabolism by phosphorylating specific proteins. Whilst it is clear that activation of this enzyme can induce an exocytotic response in several cell types[3] its role in the regulation of fluid secretion is not clear[1,2,4]. Phorbol esters, such as 12-0-tetradecanoyl phorbol-13-acetate (TPA) are potent, exogenous activators of protein kinase C[4] and so, in the present experiments, we have explored the effects of this compound upon the cholinergic regulation of membrane potassium permeability in superfused pieces of rat submandibular gland.

Methods

Submandibular glands were removed from freshly killed Wistar rats and placed in ice cold, physiological saline solution (composition in mmol l^{-1}: NaCl, 103; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; $NaHCO_3$, 25; NaH_2PO_4, 1.15; D-glucose, 2.8; sodium fumarate, 2.7; sodium glutamate, 4.9; sodium pyruvate, 4.9). The glands were chopped into fragments (< 0.5 mm), loaded with $^{86}Rb^+$ and 30–50 mg of tissue pieces transferred to a flow chamber where they were superfused (37 °C, 2 ml min^{-1}) with unlabelled saline. Rate constants for the subsequent efflux of $^{86}Rb^+$ were determined as detailed elsewhere[5,6]. In all experiments the glandular tissue was initially superfused with a nominally calcium-free solution. Our earlier experiments showed that complex effects could develop if rat submandibular tissue was exposed to solutions in which $[Ca^{2+}]_0$ had been reduced to very low levels by adding calcium-chelating agents. The calcium-free solution used in the present experiments was therefore prepared by simply omitting $CaCl_2$ from the nomal solution. The concentration of ionised calcium such as 'calcium-free' solution is ~ 20 μmol l^{-1} (ref. 7), but we have shown that the response to acetylcholine is not sustained under these conditions and this suggests that no net influx of calcium occurs[5,8].

Acetylcholine (10^{-5} mol l^{-1}) was added directly to the calcium-free solution and $[Ca^{2+}]_0$ was then elevated to 2 mmol l^{-1} as indicated on the figure. The basal rate of $^{86}Rb^+$-efflux was defined as the mean rate constant measured over the 3 min immediately prior to addition of acetylcholine and responses to acetylcholine were quantified by subtracting basal efflux from the rate of efflux measured at the peak of the response. Calcium-evoked increases in $^{86}Rb^+$-efflux were similarly quantified using the rate constant measured immediately prior to elevation of $[Ca^{2+}]_0$. Data are presented as means ± SE (n = number of experiments) and the significance

of any differences between means were tested either by analysis of variance or using Student's unpaired *t*-test.

Results

Under nominally calcium-free conditions acetylcholine increased the rate of $^{86}Rb^+$-efflux. The response reached a peak ($\Delta min^{-1} = 0.026 \pm 0.003$, n = 11) after 1 min but there was then a rapid decline to the basal level (fig., A). Subsequent elevations of $[Ca^{2+}]_0$, in the continued presence of acetylcholine, evoked further rises in the rate of $^{86}Rb^+$-efflux (fig.). The response to the first such elevation consisted of a rapid increase in efflux rate to a peak value ($\Delta min^{-1} = 0.029 \pm 0.004$, n = 11), from which there was a decline to a sustained phase that persisted until $[Ca^{2+}]_0$ was returned to low levels (fig.). Subsequent elevations of $[Ca^{2+}]_0$ evoked further increases in $^{86}Rb^+$-efflux but these responses (second: $\Delta min^{-1} = 0.014 \pm 0.002$, third: $\Delta min^{-1} = 0.012 \pm 0.002$) were smaller ($p < 0.01$ and $p < 0.001$ respectively, analysis of variance) than the initial response (fig., A).

The effects of TPA upon these calcium-dependent responses was investigated by including this compound (20 nmol l^{-1}) in the superfusing saline from 1.5 min after the initial application of acetycholine (fig., B). The response to the first elevation of $[Ca^{2+}]_0$ ($\Delta min^{-1} = 0.020 \pm 0.003$, n = 6) was slightly smaller than control but this effect was not statistically significant. Subsequent responses (second: Δmin^{-1} 0.013 ± 0.001, third: Δmin^{-1} 0.011 ± 0.002) also could not be distinguished from control. Essentially identical data were obtained when the tissue was exposed to a higher concentration (80 nmol l^{-1}) of TPA (fig., B). TPA did not, therefore, inhibit the $[Ca^{2+}]_0$-dependent component of the response to acetylcholine.

In subsequent experiments glandular tissue was superfused with TPA-containing (20 nmol l^{-1}) saline for 5 min prior to addition of acetylcholine. The transient

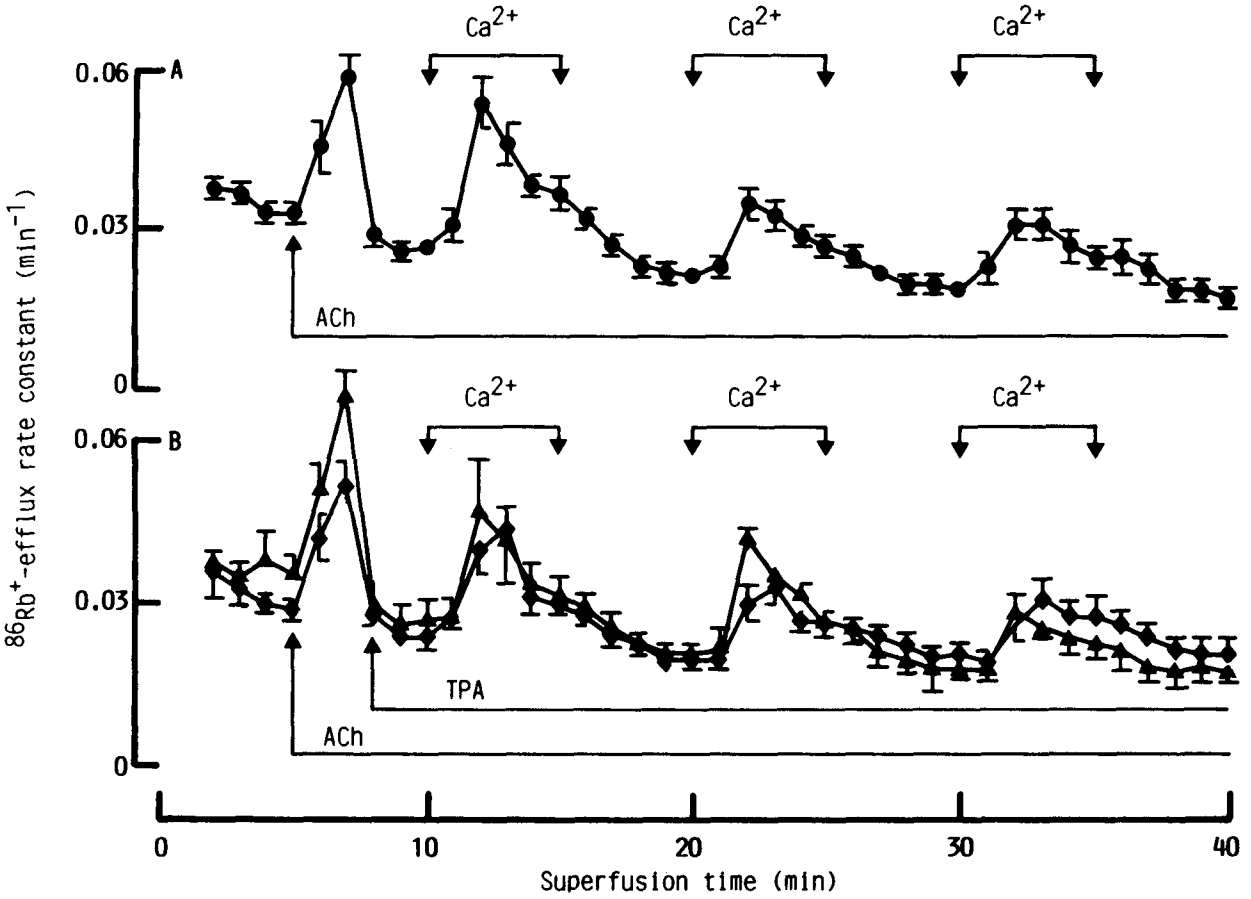

Figure. Effects of TPA upon the $[Ca^{2+}]_0$ – dependent component of the response to acetylcholine. Rate constants for $^{86}Rb^+$-efflux have been plotted against superfusion time. Data points are mean values and vertical bars show the standard error of each mean. Glandular tissue fragments were initially superfused with a nominally calcium-free solution to which 10^{-5} mol l^{-1} acetylcholine was added as indicated. $[Ca^{2+}]_0$ was then elevated to 2 mmol l^{-1} for each of the indicated periods. A freshly prepared acetylcholine-containing solution was added to each solution change to ensure that degredation of this compound did not occur during the course of each experiment.

A shows data obtained in control experiments (n = 11) where *B* shows the effects of 20 nmol l^{-1} (▲, n = 6) and 80 nmol l^{-1} (◆ n = 3) TPA.

Table 1. Effects of TPA upong the basal rate of $^{86}Rb^+$-efflux (min^{-1}) and the transient and sustained components of the acetylcholine-evoked increase in the rate of isotope efflux (Δmin^{-1}).

	Control (n = 8)	TPA (n = 6)
Basal efflux	0.033 ± 0.002	0.027 ± 0.002
Transient	0.024 ± 0.003	0.014 ± 0.003*
Sustained	0.024 ± 0.004	0.022 ± 0.006

Cells were initially sperfused with nominally calcium-free solution to which 20 nmol l^{-1} TPA was added. Five minutes later the cells were exposed to 10^{-5} mol l^{-1} acetylcholine and after a further 5 min $[Ca^{2+}]_0$ was elevated to 2 mmol l^{-1}. The resultant increases in $^{86}Rb^+$-efflux were quantified (see 'Methods') and are tabulated along with control data obtained in a parallel series of experiments.
*$p < 0.001$ with respect to the control group.

$[Ca^{2+}]_0$-independent response observed under these conditions was smaller than that evoked in parallel, control experiments although the subsequent $[Ca^{2+}]_0$-dependent increase in efflux was normal (table). TPA thus appears to inhibit the $[Ca^{2+}]_0$-independent components of the response to acetylcholine.

Discussion

Changes in the rate of $^{86}Rb^+$-efflux from preloaded pieces of glandular tissue indicate changes in cellular potassium permeability provided that the cytoplasmic $^{86}Rb^+/K^+$ ratio is low [6,9]. This requirement is satisfied by the present experimental conditions[5] and so our control data confirm that acetylcholine increases cellular potassium permeability in the rat submandibular gland. This response typically consists of a transient, $[Ca^{2+}]_0$-indepedent phase and a sustained, $[Ca^{2+}]_0$-dependent element and these components of the response could be attributed to the mobilisation of internal calcium and to receptor-regulated calcium influx respectively[5,6].

An earlier study of the perfused rabbit submandibular gland indicated that TPA specifically inhibited the sustained phase of acetylcholine-evoked fluid secretion[10]. This part of the response to acetylcholine was acutely dependent upon $[Ca^{2+}]_0$ and so these authors suggested that exogenous activation of protein kinase C may limit the influx of calcium into stimulated cells[10]. In the present experiments, however, TPA had no effect upon the sustained $[Ca^{2+}]_0$-dependent component of this response to acetylcholine but this compound did inhibit the initial $[Ca^{2+}]_0$-independent phase. Exogenous activation of protein kinase C may thus impair the acetylcholine-evoked mobilisation of calcium from internal stores but does not appear to inhibit the influx of calcium into stimulated cells.

Experiments in which $[Ca^{2+}]_i$ was monitored directly suggested that TPA may exert different effects on acinar cells from different species[11]. In murine lacrimal acini this compound thus caused substantial inhibition of the response to brief pulses of acetylcholine but it had essentially no effect in rabbit submandibular acini. There may thus be species-specific differences in the sensitivity of the stimulus-secretion coouppling mechanism to activators of protein kinase C[11]. Vincentini et al.[12] showed that exogenous activation of protein kinase C inhibited cholinergically-evoked increases in $[Ca^{2+}]_i$ but that this compound had no effect upon the affinity or density of the muscarnic receptors on the cell surface. Biochemical[3,13,14], electrophysiological[15,16] and microspectrofluorimetric[17,18] studies have similarly suggested that TPA impairs the receptor-regulated hydrolysis of PIP_2. It has therefore been suggested that the production of diacylglycerol, the endogenous activator of protein kinase C, within the membrances of stimulated cells may allow calcium mobilising agonists to regulate the activity of protein kinase C. This enzyme, in turn, may exert inhibitory control over the entire signal transduction pathway and this would provide a physiological basis for such phenomena as the desensitisation that developes during prolonged stimulation[3,4,13–16].

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