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EFFECTS OF THEOPHYLLINE AND DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE ON THE MEMBRANE POTENTIAL OF MOUSE PANCREATIC β-CELLS

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SUMMARY

1. The effects of theophylline and dibutyryl cyclic AMP on the membrane potential of mouse β-cells were studied with micro-electrodes. They were compared to their effects on insulin release by perifused mouse islets.

2. In 3 mM-glucose, theophylline (10 mM) depolarized the β-cell membrane and stimulated insulin release, but did not induce electrical activity. Dibutyryl cyclic AMP (1 mM) was without effect.

3. In 7 mM-glucose, theophylline (0·5–2 mM) and dibutyryl cyclic AMP (1 mM) slightly depolarized the β-cell membrane, induced electrical activity in otherwise silent cells and increased insulin release. A higher concentration of theophylline (10 mM) hyperpolarized the β-cell membrane, did not induce electrical activity, but also stimulated insulin release.

4. In 10 mM-glucose, the membrane potential of β-cells exhibited repetitive slow waves with bursts of spikes on the plateau. Under steady state, these slow waves were differently affected by low or high concentrations of theophylline. At 0·5–2 mM, theophylline shortened the intervals, lengthened the slow waves and slightly increased their frequency. On the other hand, 10 mM-theophylline markedly decreased the duration of both intervals and slow waves, and increased their frequency. The effects of 1 mM-dibutyryl cyclic AMP were similar to those of 2 mM-theophylline.

5. With 2–10 mM-theophylline, two other effects were also observed: a transient hyperpolarization with suppression of electrical activity immediately after addition of the methylxanthine and an increase in electrical activity upon its withdrawal.

6. Theophylline and dibutyryl cyclic AMP markedly potentiated insulin release induced by 10 mM-glucose. The magnitude of these changes did not correlate well with the importance of the changes in electrical activity. However, with 2–10 mM-theophylline the increase in release was also preceded by an initial transient inhibition, whereas withdrawal of the methylxanthine was accompanied by a further increase.

7. When Ca influx was inhibited by D600, the slow waves were suppressed, the membrane was depolarized to the plateau level and only few spikes were present. Although theophylline markedly increased insulin release under these conditions, it did not affect the membrane potential.

8. Several conclusions can be drawn from this study. Insulin release and electrical activity in β-cells can be dissociated when intracellular Ca is used to trigger exocytosis. High concentrations of theophylline produce effects unrelated to cyclic AMP. The ionic effects of theophylline and dibutyryl cyclic AMP are not restricted to mobilization of cellular Ca, but also involve changes in the ionic (K, Ca) permeabilities of the β-cell membrane. Cyclic AMP may play an important role in the control of the electrical activity induced by glucose in insulin-secreting cells.

INTRODUCTION

Considerable attention has been focused on the role of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) in the control of insulin release. It is now widely accepted (Montague & Howell, 1975; Sharp, 1979; Hedeskov, 1980) that cyclic AMP is not the key intermediate in the stimulus–secretion coupling in pancreatic β-cells, but that it can potentiate the releasing effect of a primary stimulus. Thus, insulin secretion triggered by glucose or other secretagogues is enhanced, whenever the concentration of cyclic AMP in β-cells is increased by activators of the adenylate cyclase (e.g. glucagon), by phosphodiesterase inhibitors (e.g. methylxanthines) or by dibutyryl cyclic AMP. The mechanisms by which cyclic AMP potentiates insulin release are still incompletely understood, but much evidence has accumulated, indicating the crucial importance of the interactions between Ca and cyclic AMP. The nucleotide is believed to amplify the rise in cytoplasmic free Ca^{2+} brought about by the stimulus, either by mobilizing intracellular bound Ca or by preventing Ca sequestration in cellular organelles (Brisson, Malaisse-Lagae & Malaisse, 1972; Wollheim & Sharp, 1981). It is currently thought to exert no direct effect on the ionic permeabilities of the β-cell membrane.

Concentrations of glucose that stimulate insulin release induce membrane depolarization and electrical activity in pancreatic β-cells (Dean & Matthews, 1970; Meissner & Schmelz, 1974). The correlations between the glucose dependence or the time course of the changes in electrical activity in single β-cells and in insulin release by whole islets strongly suggest a causal relationship between both events (Meissner, 1976*a*; Meissner & Atwater, 1976; Meissner & Preissler, 1979; Scott, Atwater & Rojas, 1981). The validity of the proposal has been verified under many experimental conditions. Surprisingly, however, very little attention has been paid to the changes in membrane potential or in electrical activity during potentiation of insulin release by agents raising cyclic AMP levels in β-cells. Only two abstracts have appeared, which report that theophylline may increase glucose-induced electrical activity at low concentration (Meissner, 1976*b*) or decrease it at high concentration (Atwater & Ribalet, 1979). In these experiments we have studied the effects of theophylline, a widely used inhibitor of the cyclic AMP phosphodiesterase, on the membrane potential of single mouse β-cells. These effects were compared to those of dibutyryl cyclic AMP, to discriminate between changes mediated by the rise in cellular cyclic AMP and changes due to the methylxanthine itself. Insulin release by perifused mouse islets was also measured to gain a better insight into the links between the changes in membrane potential and in insulin release. A brief account of part of the study has been published previously (Henquin & Meissner, 1982*b*).

METHODS

All experiments were performed with islets of fed female NMRI mice. The animals received an intraperitoneal injection of pilocarpine, 20 mg/kg, before being killed. Such treatment facilitates visualization and isolation of the islets without affecting the characteristics of their electrical and secretory responses to glucose stimulation. For electrophysiological records, a piece of pancreas was fixed in a small perifusion chamber (1 ml) and the membrane potential of single β-cells was continuously recorded with micro-electrodes. The method has been described in detail (Meissner & Schmelz, 1974). β-cells were identified by the typical electrical activity (Meissner, 1976*a*) that they display in the presence of 10–15 mM-glucose. The criteria for a successful impalement have been defined previously (Henquin & Meissner, 1982*a*). For measurement of insulin release, groups of twelve or forty islets, isolated by collagenase digestion of the pancreas, were placed in perifusion chambers (Henquin, 1978, 1979). Immunoreactive insulin was assayed in the effluent fractions, using mouse insulin as standard (Novo Research Institute, Bagsvaerd, Denmark).

The perifusion medium had the following ionic composition (mM): NaCl, 122; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 20. It was continuously gassed with a mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7·4, and the temperature was kept at 37 °C. In experiments measuring insulin release, the medium was also supplemented with bovine serum albumin, 5 mg/ml. Test substances were obtained from the following sources: theophylline from Merck A.G. (Germany), isobutylmethylxanthine from Aldrich Europe (Belgium), dibutyryl cyclic AMP from Sigma Chemical Co. (U.S.A.) and D600 from Knoll A.G. (Germany).

Results are presented by representative experiments or, whenever possible, as means ± S.E. of the mean.

RESULTS

Effects of methylxanthines and dibutyryl cyclic AMP in the presence of a non-stimulatory concentration of glucose

In the presence of 3 mM-glucose, the membrane potential of mouse β-cells was stable and averaged −68 mV; no electrical activity was present. Theophylline (10 mM) or isobutylmethylxanthine (1 mM) depolarized the membrane (Table 1). Their effect started approximately after 1 min, usually reached a steady state after 6–7 min, and was reversible upon withdrawal of the methylxanthines. It was consistently smaller than the difference between the resting and the threshold membrane potential at which glucose-induced activity started (12·8 ± 0·8 mV). Neither theophylline nor isobutylmethylxanthine induced electrical activity in β-cells at 3 mM-glucose. On the other hand, the rate of insulin release was clearly increased by the methylxanthines under these conditions (Table 1). By contrast, addition of 1 mM-dibutyryl cyclic AMP to a perifusion medium containing 3 mM-glucose had no effect on the resting membrane potential or basal insulin release (Table 1).

Effects of theophylline and dibutyryl cyclic AMP in the presence of a threshold concentration of glucose

Fig. 1 illustrates the effect of theophylline on the membrane potential of a mouse β-cell perifused with 7 mM-glucose. In this cell, no electrical activity was induced by 7 mM-glucose alone. Addition of 0·5 mM-theophylline caused a gradual depolarization and appearance of slow waves of membrane potential, with spikes superimposed on the plateau (Fig. 1*A*). This electrical activity was similar to that induced by a higher concentration of glucose. If theophylline was added at a concentration of 2 mM, the β-cell membrane first hyperpolarized before depolarizing slowly until slow waves appeared (Fig. 1*B*). In the presence of an even higher concentration of theophylline

TABLE 1. Effects of methylxanthines and dibutyryl cyclic AMP on the membrane potential of mouse β-cells and on insulin release by mouse islets perifused with 3 mM-glucose

Test substance (mM)	Change in membrane potential (mV)	Electrical activity (No. of cells)	Insulin release (pg/min . islet) 10 min	30 min
—	(−68·0 ± 2·0)	0/11	19 ± 1 (13)	
Theophylline (10)	−5·8 ± 1·0*	0/4	51 ± 2**	53 ± 3 (5)**
Isobutylmethylxanthine (1)	−8·0 ± 1·4*	0/4	67 ± 5**	76 ± 4 (5)**
Dibutyryl cyclic AMP (1)	−0·7 ± 0·9	0/3	19 ± 2	18 ± 2 (3)

The membrane potential was measured in the indicated number of cells (different mice). The control value corresponds to the average of the absolute membrane potentials measured during the last minute preceding addition of the test substances (for 10–16 min). The effect of these test substances is given as the maximum change (minus sign means depolarization) from the control value in the same cell. Electrical activity is defined as slow waves of membrane potential with superimposed spikes. Insulin release was measured in a number of separate experiments indicated in parentheses. The values are the absolute rates of insulin release before and 10 or 30 min after addition of the test substances. Values are means ± S.E. of the mean. Statistical significance (paired t test) of the effect of test substance: * $P < 0.02$; ** $P < 0.005$.

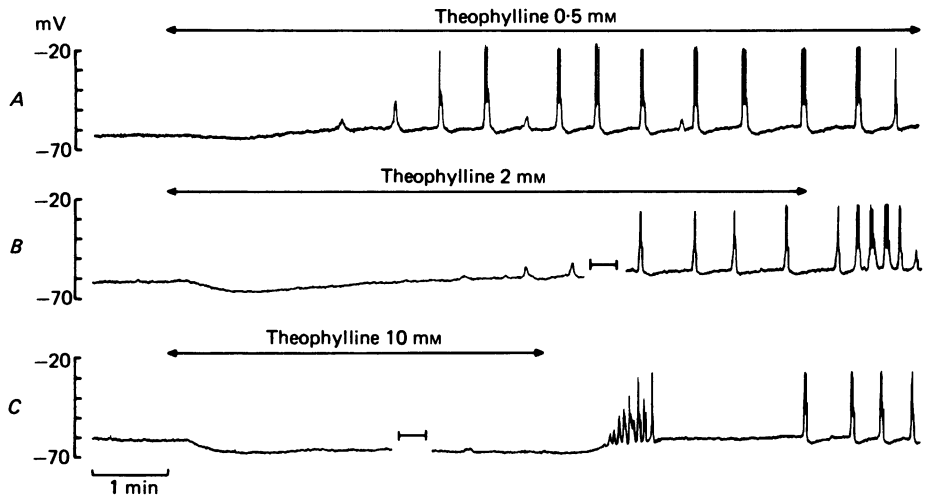

Fig. 1. Effect of theophylline on the membrane potential of a single mouse β-cell perifused with 7 mM-glucose. Theophylline was added, at the indicated concentration, for a period of 20 min; the intervals not shown in records B and C were of 12 and 15·5 min, respectively. The three records were obtained in the same cell in the order A, C, B with a period of at least 15 min in the presence of 7 mM-glucose alone between additions of theophylline. These records are representative of six to eight different experiments.

(10 mM), the membrane hyperpolarized persistently and did not exhibit electrical activity (Fig. 1C). However, removal of the methylxanthine was accompanied by a depolarization and the appearance of slow waves. When the concentration of theophylline was 2 mM, its withdrawal from the medium was followed by a marked increase in the duration and frequency of the slow waves (Fig. 1B). No such 'off' response was seen after 0·5 mM-theophylline.

Table 2 summarizes the results obtained in fourteen cells (different mice) studied in the presence of 7 mM-glucose. The average membrane potential was less negative

($P < 0.005$) than in the presence of 3 mM-glucose (Table 1); electrical activity was present in one-third of the cells. The β-cell membrane hyperpolarized during the first 2 min following addition of 2 or 10 mM-theophylline. Later on, it depolarized in the presence of 0·5 or 2 mM-theophylline, but remained hyperpolarized with 10 mM-theophylline.

Addition of theophylline to a medium containing 7 mM-glucose was followed by a delayed (5–6 min) and progressive increase in insulin release (Fig. 2). The rate of

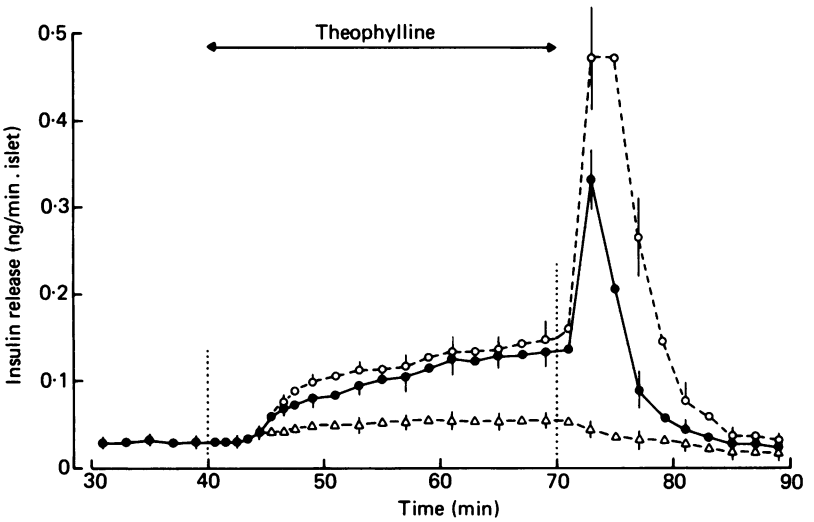

Fig. 2. Effect of theophylline on insulin release by mouse islets perifused with 7 mM-glucose. Theophylline (0·5 mM, △; 2 mM, ● and 10 mM, ○) was added between 40 and 70 min. Values are means ± S.E. of the mean of five experiments.

TABLE 2. Effects of theophylline and dibutyryl cyclic AMP on the membrane potential of mouse β-cells perifused with 7 mM-glucose

Test substance (mM)	Change in membrane potential (mV) 0–2 min	Change in membrane potential (mV) > 7 min	Electrical activity (No. of cells)	Fraction of plateau phase
—	(−60·2 ± 1·3)		5/14	0·012 ± 0·005
Theophylline (0·5)	0 ± 0·8	−3·6 ± 0·8**	6/7	0·071 ± 0·014**
Theophylline (2)	+3·9 ± 0·7**	−2·9 ± 0·5*	7/8	0·054 ± 0·010*
Theophylline (10)	+7·7 ± 0·9***	+4·0 ± 0·7**	0/6	0
Dibutyryl cyclic AMP (1)	−0·8 ± 0·5	−4·2 ± 0·6**	5/5	0·200 ± 0·015***

The membrane potential was measured in the indicated number of cells (different mice). The control value corresponds to the average of the absolute membrane potentials measured during the last minute preceding addition of the test substances (for 10–30 min). The different test substances were sometimes tested in the same cell. Their effect is given as the change from the control value in the same cell. This change was measured during the first 2 min or at least 7 min after addition of the test substance. Minus sign means depolarization; plus sign means hyperpolarization. Electrical activity is defined as slow waves of membrane potential with superimposed spikes. The mean fraction of plateau phase was calculated for all cells, including those which did not show electrical activity (= 0). Values are means ± S.E. of the mean. Statistical significance (paired t test) of the effect of test substance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

secretion was approximately doubled with 0·5 mM-theophylline and potentiated 3·5-fold with 2 or 10 mM-theophylline (Fig. 2). There is thus a discrepancy between the increase in release and in electrical activity, estimated by the fraction of plateau phase in single β-cells (Table 2). This latter was highest with the lowest concentration of theophylline tested. Furthermore, approximately the same rate of release was measured in the presence of 2 or 10 mM-theophylline although electrical activity was almost always present with 2 mM- and never seen with 10 mM-theophylline (Table 2).

Upon removal of theophylline, an increase in insulin release occurred, if the methylxanthine was tested at 10 or 2 mM, but not at 0·5 mM (Fig. 2). These 'off' responses were observed in all experiments and correspond to the increase in electrical activity recorded after removal of 2 mM-theophylline (four of eight cells) or 10 mM-theophylline (six of six cells).

Addition of 1 mM-dibutyryl cyclic AMP to a medium containing 7 mM-glucose depolarized the β-cell membrane and induced electrical activity in all cells. It increased the fraction of plateau phase markedly (Table 2) and augmented insulin release approximately 4-fold. These effects of dibutyryl cyclic AMP have recently been reported in greater detail (Henquin & Meissner, 1983).

Effects of theophylline and dibutyryl cyclic AMP in the presence of a stimulatory concentration of glucose

In the presence of 10 mM-glucose, the membrane potential of β-cells exhibited repetitive slow waves with a fast spike activity originating from the plateau level (Meissner, 1976*a*). The initial effect of theophylline was a slight hyperpolarization of the β-cell membrane with transient suppression of electrical activity (Fig. 3*A* and *C*). Both the amplitude and the duration of this hyperpolarization were dose-dependent. When the slow waves resumed, their appearance was markedly influenced by the concentration of theophylline (Fig. 3*B* and *D*). The characteristics of the changes induced by the methylxanthine will be described in detail in a later section. Upon withdrawal of theophylline, an increase in electrical activity was consistently observed when the methylxanthine was used at concentrations of 2 or 10 mM (Fig. 3*B* and *D*), but not at 0·5 mM. The importance of this 'off' response was more marked after 10 than after 2 mM-theophylline.

In contrast to the methylxanthine, dibutyryl cyclic AMP (1 mM) did not hyperpolarize the β-cell membrane nor increase the intervals between the slow waves (Fig. 4). In certain cells, however, the amplitude of the spikes decreased for 1 or 2 min (Fig. 4*B*). Later on the duration of the slow waves increased markedly (see later section), and sometimes the membrane remained depolarized at the plateau level and exhibited a continuous spike activity (Fig. 4*B*). Withdrawal of dibutyryl cyclic AMP was not accompanied by an increase in electrical activity, but only followed by a slow return to the pattern recorded before addition of the nucleotide (not shown).

The effect of theophylline on glucose-induced insulin release is illustrated in Fig. 5. The rate of secretion measured in the presence of 10 mM-glucose alone (107 ± 3 pg/min . islet) was 5-fold higher ($P < 0{\cdot}001$) than that measured in the presence of 3 mM-glucose. Addition of 2 mM-theophylline to the perifusion medium

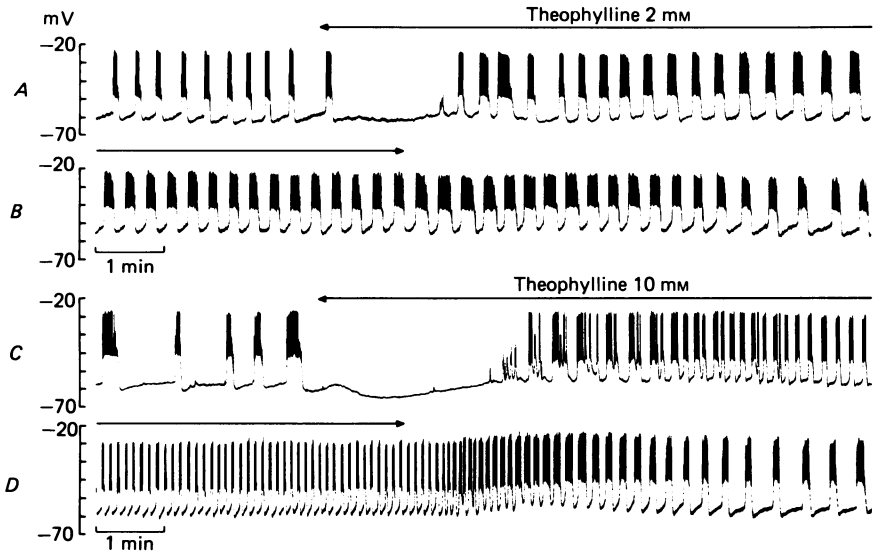

Fig. 3. Effect of theophylline on the membrane potential of single mouse β-cells perifused with 10 mM-glucose. Theophylline was added, at the indicated concentrations, for a period of 30 min. Record *B* is the continuation of *A* and record *D* is the continuation of *C*, with an interval of 17·5 min. The two concentrations of theophylline were tested in two different cells. These records are representative of five and four experiments.

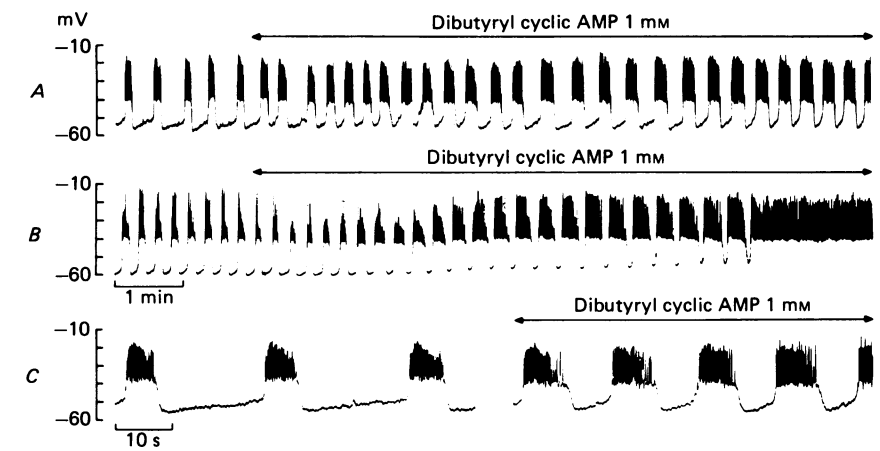

Fig. 4. Effect of dibutyryl cyclic AMP on the slow waves of membrane potential triggered by 10 mM-glucose in mouse β-cells. The concentration of glucose was 10 mM throughout and dibutyryl cyclic AMP (1 mM) was added as indicated by the arrows. Records *A* and *B* were obtained in different cells. *C* shows, on a faster time scale, details of record *A* without and with dibutyryl cyclic AMP.

caused an initial transient decrease in insulin release, followed by a marked potentiation. Withdrawal of the methylxanthine was accompanied by a further increase in secretion rates before their return to control levels (Fig. 5). A similar 'off' response has been described previously in the perfused rat pancreas (Matschinsky, Landgraf, Ellerman & Kotler-Brajtburg, 1972). The initial decrease, albeit small, was

consistently seen and statistically significant 2 and 3 min after addition of 2 mM-theophylline ($P < 0.05$ by paired t test) and 2, 3 and 4 min after addition of 10 mM-theophylline ($P < 0.005$). The 'off' response observed upon removal of theophylline was much larger when the concentration of the methylxanthine was 10 mM than 2 mM (not shown). A lower concentration of theophylline (0·5 mM) and dibutyryl cyclic AMP (1 mM) only potentiated glucose-induced insulin release without producing an initial inhibition nor an 'off' response when they were removed from the perifusion medium (not shown).

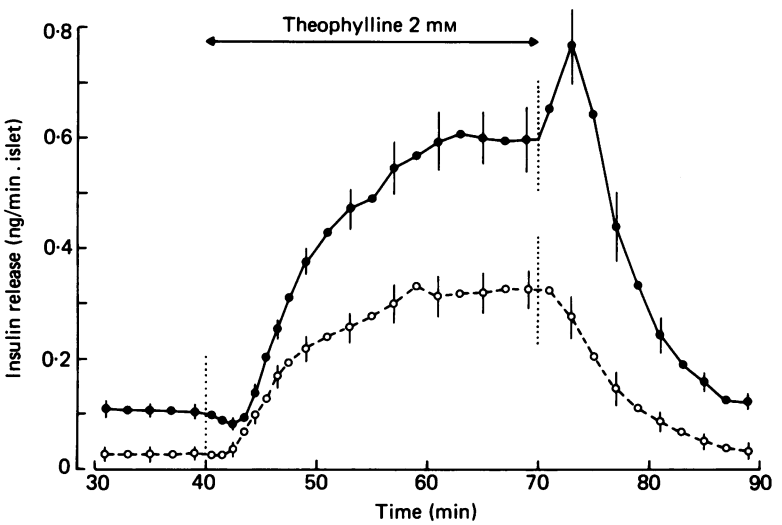

Fig. 5. Effect of theophylline on insulin release by mouse islets perifused with 10 mM-glucose. In one series (○) the medium was supplemented with 50 μM-D600. Theophylline (2 mM) was added between 40 and 70 min. Values are means ± S.E. of the mean of seven experiments without and six experiments with D600.

Fig. 6 compares the increase in electrical activity in β-cells and in insulin release from isolated islets triggered by various concentrations of theophylline and by dibutyryl cyclic AMP in the presence of 10 mM-glucose. The intensity of electrical activity was estimated as the fraction of plateau phase, i.e. the fraction of time spent at the depolarized plateau level, with spike activity. All concentrations of theophylline tested increased the fraction of plateau phase, but the effect was not dose-dependent (Fig. 6, left panel): it was similar with 0·5 or 2 mM-theophylline and much less marked with 10 mM-theophylline. The highest increase was recorded in the presence of 1 mM-dibutyryl cyclic AMP. The potentiation of insulin release was also greatest with dibutyryl cyclic AMP, but the effect of theophylline was more dose-dependent. It was smallest with 0·5 mM-theophylline and several-fold larger with 2 or 10 mM-theophylline (not different from each other) (Fig. 6, right panel). The time course of the increase in electrical activity and in insulin release was also clearly different. The potentiation of insulin release augmented progressively and reached a maximum after 30 min, whereas, with dibutyryl cyclic AMP and 0·5 or 2 mM-theophylline, the maximum increase in electrical activity was already recorded after 10 min.

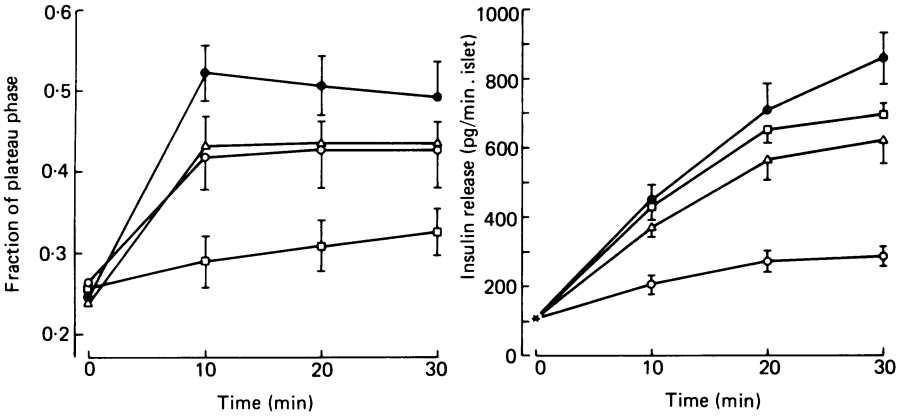

Fig. 6. Effect of 1 mM-dibutyryl cyclic AMP (●) and of various concentrations of theophylline (0·5 mM, ○; 2 mM, △ and 10 mM, □) on the fraction of plateau phase in single β-cells and on insulin release by isolated islets. The concentration of glucose was 10 mM throughout the experiments. The test substances were added for 30 min. In each experiment, the fraction of plateau phase, i.e. the fraction of time spent at the depolarized plateau level, with spike activity, was measured during the periods 8–10, 18–20 and 28–30 min. The control value was measured during the 2 min preceding addition of the test substance. Insulin release was measured in separate experiments, with batches of twelve islets. The control rate of release for all experiments was 107 ± 3 pg/min . islet. Values are means ± S.E. of the mean for four to five cells (fraction of plateau phase) and for four to seven batches of perifused islets (insulin release).

Effects of theophylline and dibutyryl cyclic AMP on the slow waves of membrane potential induced by glucose

Typical slow waves induced by 10 mM-glucose in a control medium or with theophylline or dibutyryl cyclic AMP are shown, on a fast time scale, in Figs. 7 and 4*C*. The changes produced by the test agents on the various phases of the slow waves have been measured in four or five cells and are compiled in Fig. 8. These measurements were made 10, 20 and 30 min after stimulation with theophylline or dibutyryl cyclic AMP.

The duration of slow waves (plateaux with spikes) increased with dibutyryl cyclic AMP and with 0·5 or 2 mM-theophylline, but decreased considerably in the presence of 10 mM-theophylline. A mirror image was found for the slope of the repolarization phase, which terminates the slow waves: it increased with 10 mM-theophylline, but decreased under the other experimental conditions (Fig. 8). Theophylline caused a dose-dependent shortening of the intervals between the slow waves; dibutyryl cyclic AMP also decreased them approximately to the same extent as 2 mM-theophylline. The slope of the prepotential, i.e. the progressive depolarization during the interval, was not modified by 0·5 mM-theophylline, approximately doubled (at 30 min) by 2 mM-theophylline or 1 mM-dibutyryl cyclic AMP and markedly increased by 10 mM-theophylline (Fig. 8). These modifications in the slope of the prepotential led to corresponding changes in the frequency of the slow waves: no difference with 0·5 mM-theophylline, a slight increase (approx. 45%) with 2 mM-theophylline or 1 mM-dibutyryl cyclic AMP and a marked increase (approx. 350%) with 10 mM-theophylline (Fig. 7).

Dibutyryl cyclic AMP and theophylline, at concentrations of 0·5 or 2 mM, produced no marked change in the characteristics of the spikes recorded in the presence of 10 mM-glucose. Their frequency was not affected at the onset of the slow waves, but often dropped closer to the end of the slow waves (Fig. 7). The presence of spikes of longer duration and less regular shape than under control conditions was also observed, particularly after long periods of stimulation. In certain cells where the spike amplitude was relatively small (10–15 mV), theophylline or dibutyryl cyclic AMP increased it (not shown). The most consistent alteration of the spikes was caused by 10 mM-theophylline: their frequency was reduced, but their duration was increased and their shape was often multiphasic (Fig. 7*C*).

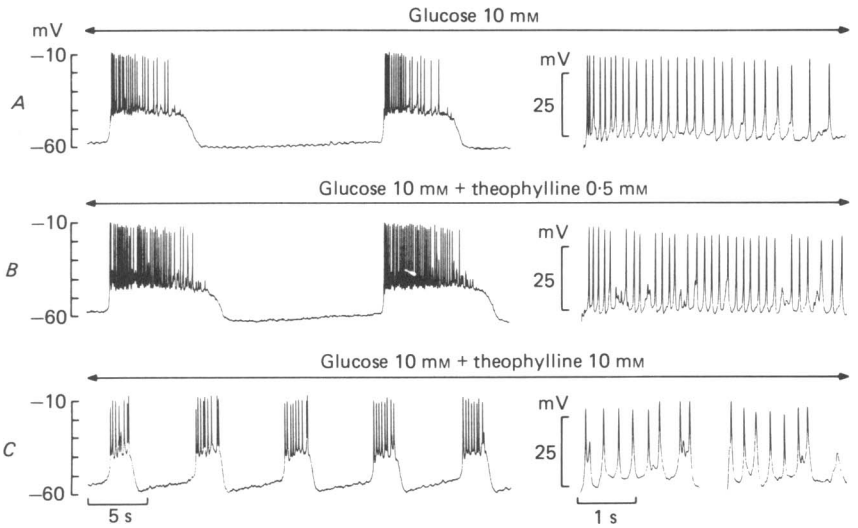

Fig. 7. Effect of theophylline on the slow waves of membrane potential and on the spikes triggered by 10 mM-glucose in pancreatic β-cells. Records *A* and *B* are from the same cell and record *C* is from another cell. Records *B* and *C* were obtained 10 min after addition of theophylline. To the right are shown, on a faster time scale and with a higher voltage gain, the spikes originating from the plateau level of one (*A* and *B*) or two (*C*) slow waves.

Effects of theophylline in the presence of a high concentration of glucose

When the concentration of glucose is raised from 10 to 15 mM, the slow waves lengthen and the intervals shorten (Meissner, 1976*a*). Addition of theophylline to a medium containing 15 mM-glucose transiently (1–2 min) decreased the duration of the slow waves, with little effect on the intervals. Thereafter, the electrical activity increased. The three concentrations of theophylline produced changes in slow waves and intervals, qualitatively similar to those described in detail for the experiments carried out with 10 mM-glucose. The only significant difference was that, in six of seven cells, 2 mM-theophylline caused a persistent depolarization with continuous spike activity after about 8–10 min. From these observations in the presence of a high concentration of glucose and the evidence that neither theophylline nor dibutyryl cyclic AMP affects glucose metabolism in mouse islets (Ashcroft, Weerasinghe, Bassett & Randle, 1972; Henquin & Meissner, 1983), it appears unlikely that a mere

acceleration of metabolic fluxes accounts for the effects of the methylxanthine and the nucleotide on the membrane potential of β-cells.

Effects of theophylline in the presence of D600

In rat islets, D600 inhibits glucose-stimulated insulin release by blocking Ca influx (Malaisse, Devis, Pipeleers & Somers, 1976) and theophylline partially overcomes the inhibition of release (Henquin, 1978) without correcting the inhibition of Ca uptake (Henquin, Charles, Nenquin, Mathot & Tamagawa, 1982). In mouse islets also, D600 inhibited glucose-stimulated insulin release (Fig. 5), but theophylline remained able to increase the secretion rate markedly. In the presence of D600, however, no 'off' response occurred upon withdrawal of the methylxanthine.

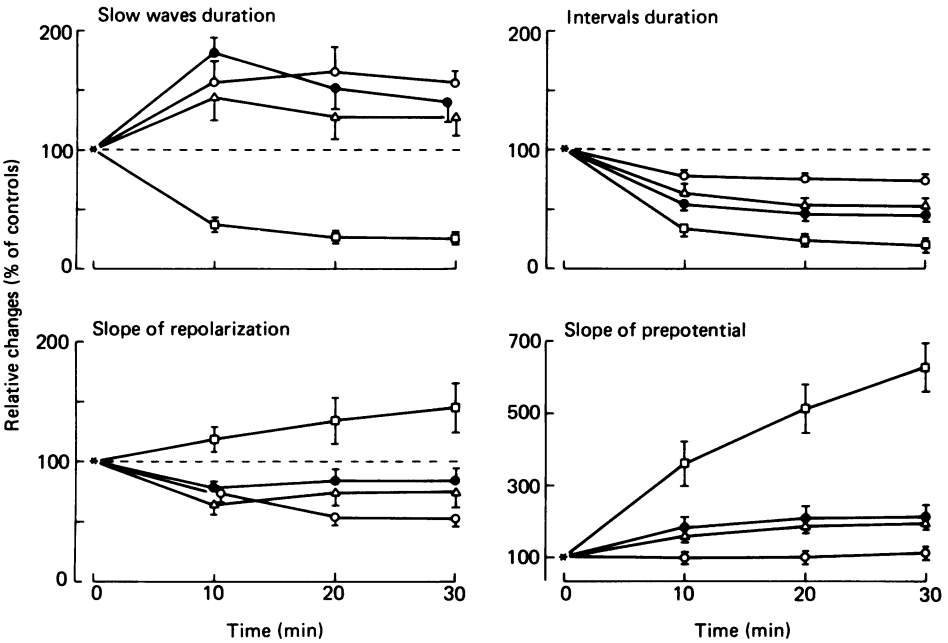

Fig. 8. Effect of 1 mM-dibutyryl cyclic AMP (●) and of various concentrations of theophylline (0·5 mM, ○; 2 mM, △ and 10 mM, □) on the slow waves of membrane potential triggered by 10 mM-glucose in mouse β-cells. The test substances were added for 30 min. In each experiment, all slow waves and intervals were measured during the periods 8–10, 18–20 and 28–30 min. The values are expressed as percentages of the control values measured in the same cell during the 2 min immediately preceding addition of the test substance. There was no statistically significant difference between control values in the four experimental series. Absolute control values for all cells were: slow waves duration, 4·84 ± 0·27 s; intervals duration, 14·00 ± 1·05 s; slope of repolarization, 14·20 ± 1·12 mV/s; slope of prepotential, 0·29 ± 0·02 mV/s. The prepotential is defined as the progressive depolarization (during the interval), which precedes the fast depolarization of the slow wave. Values are means ± S.E. of the mean for four to five cells.

It is known that D600 inhibits glucose-induced electrical activity in β-cells (Matthews & Sakamoto, 1975). More recent studies (Ribalet & Beigelman, 1980; Meissner & Schmeer, 1981), have shown that in the presence of D600 the slow waves of membrane potential disappear and the membrane gradually depolarizes to the

plateau level. This is illustrated by Fig. 9*A*. After addition of the drug to the perifusion medium, the membrane potential stabilized at the plateau level; only a few spikes of small amplitude and low frequency were present and they tended to disappear with time. Theophylline (2 mM) had no effect; even 20 min after its addition, the membrane potential was stable and no activity was present. Furthermore, withdrawal of theophylline from the medium containing D600 failed to produce an 'off' response (Fig. 9*B*). By contrast, when the concentration of extracellular Ca was raised to 10 mM, the membrane depolarized transiently, spikes of increasing amplitude resumed and, finally, slow waves of short duration with spikes on the plateau reappeared. In certain cells, however, a small effect of theophylline could be detected despite the presence of D600 (Fig. 9*C*). When the spike activity was incompletely suppressed by D600, theophylline tended to induce small oscillations in the membrane potential and the residual spikes appeared in bursts. The progressive depolarization and decrease in spike amplitude also seen in Fig. 9*C* were not due to theophylline, but also occurred with D600 alone.

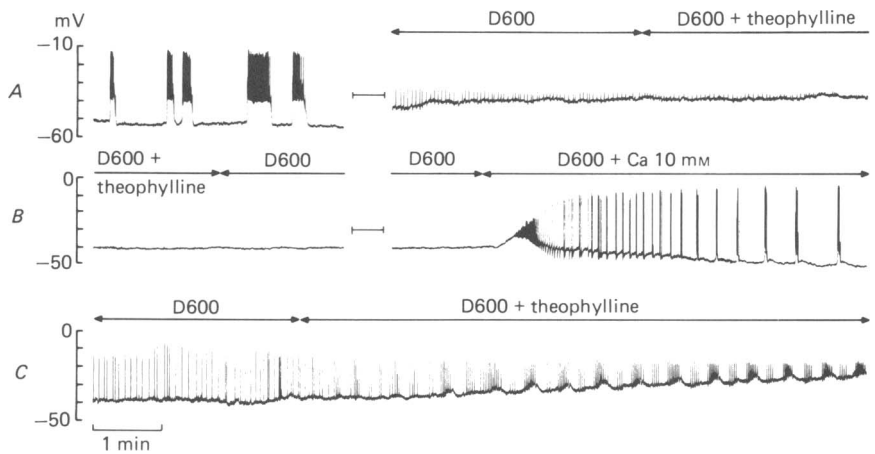

Fig. 9. Effect of theophylline on the membrane potential of single mouse β-cells perifused with 10 mM-glucose and 50 μM-D600. Records *A* and *B* were obtained in the same cell. *A*, the control activity induced by glucose alone is shown to the left and its alteration in the presence of D600 is shown to the right; the record starts 13 min after addition of D600. Theophylline (2 mM) was added and withdrawn as indicated by the arrows. *B* is the continuation of record *A* after an interval of 15 min (total time of theophylline addition was 20 min) and an interval of 5 min intervenes the two parts of record *B*. The concentration of extracellular Ca was raised from 2·5 to 10 mM as indicated by the arrows. Record *C* was obtained in another cell and starts 5 min after addition of D600 to a medium containing 10 mM-glucose. Theophylline (2 mM) was added as indicated by the arrows.

DISCUSSION

Three main conclusions emerge from the present study: (1) under particular conditions, insulin release and electrical activity in β-cells can be dissociated; (2) differences exist between the effects of dibutyryl cyclic AMP and of theophylline, which suggest that the methylxanthine may have properties of its own, unrelated to the rise in cyclic AMP levels; (3) changes in the handling of intracellular Ca, which

are currently considered as the essential ionic effect of cyclic AMP in islet cells (see Introduction), cannot account for all effects of theophylline and of dibutyryl cyclic AMP on the membrane potential of β-cells.

Dissociations and correlations between insulin release and electrical activity in β-cells

Addition of a high concentration of theophylline (10 mM) to a medium containing either a non-stimulatory concentration of glucose (3 mM) or a threshold concentration of glucose (7 mM) stimulated insulin release without inducing electrical activity in β-cells. In the former case, the rate of release was higher than in the presence of 7 mM-glucose alone, a condition in which electrical activity is present in about one-third of the cells. In the latter case, it was higher than in the presence of 10 mM-glucose alone. One may thus exclude the possibility that electrical activity was not detected simply because the stimulation of the β-cell was too weak. Conversely, one is forced to conclude that insulin release can take place without electrical activity being present in β-cells. The early suggestion (Meissner & Schmelz, 1974) that spike activity could perhaps originate from channels located in the membrane of granules undergoing exocytosis is thus no longer tenable. It is also interesting to note that insulin release could be stimulated although the β-cell membrane was not depolarized (10 mM-theophylline in the presence of 7 mM-glucose). This suggests that the depolarization of the plasma membrane is not, *per se*, an absolute requirement for the exocytotic process, even if a permissive role cannot be excluded.

Another dissociation was found when Ca^{2+} influx was blocked by D600: theophylline markedly increased insulin release without restoring electrical activity. Under these conditions, the methylxanthine does not correct the inhibition of $^{45}Ca^{2+}$ uptake produced by D600 (Henquin *et al.* 1982), and the restoration of release is thought to be due to mobilization of intracellular Ca^{2+} (Malaisse *et al.* 1976; Henquin, 1978). There is also evidence that the releasing effect of methylxanthines in the presence of low concentrations of glucose is due to their ability to mobilize intracellular Ca^{2+} (Siegel, Wollheim, Kikuchi, Renold & Sharp, 1980).

No good temporal or quantitative correlation was found between the increase in insulin release and the increase in electrical activity brought about by dibutyryl cyclic AMP or theophylline in the presence of 10 mM-glucose. One could argue that the fraction of plateau phase, i.e. the time with spike activity, is not an adequate reference and that the spike activity should also be taken into consideration. Unfortunately, this latter cannot be reliably quantified by a mere counting of the number of spikes per unit of time. Thus, the spike duration varies even under control conditions; it usually increases at the end of each slow wave. Furthermore, in the presence of dibutyryl cyclic AMP or theophylline the shape of the spikes was sometimes altered.

In marked contrast with these dissociations, good correlations were found between certain subtle changes in electrical activity and in insulin release. Addition of 2 or 10 mM-theophylline to a medium containing 10 mM-glucose consistently caused an initial suppression of electrical activity and a slight inhibition of release. On the other hand, removal of theophylline was followed by an increase in electrical activity and in insulin release. This 'off' response is probably caused by a transient activation of Ca^{2+} influx since it was suppressed by D600.

In summary, this study shows that no change in β-cell membrane potential is the consequence of insulin release. It strengthens the previous suggestions (Dean & Matthews, 1970; Meissner & Schmelz, 1974; Meissner, 1976*a*) that the electrical events occurring in the β-cell membrane play a causal role in the stimulation of release, but restricts this proposal to stimuli, the insulinotropic effect of which depends on extracellular Ca^{2+}, and hence on Ca^{2+} influx. Thus the dissociations between release and electrical activity described here were observed when the origin of Ca^{2+} triggering exocytosis is thought to be partially or exclusively intracellular. Another possibility would be that, as recently proposed for platelets (Rink, Sanchez & Hallam, 1983), insulin release is stimulated by other messengers than a rise in cellular free Ca^{2+} under these conditions. The available experimental evidence does not support this alternative, but is not sufficient to exclude it formally.

Are theophylline effects always mediated by cyclic AMP?

Methylxanthines have been widely used to inhibit the cyclic nucleotide phosphodiesterase, and the resulting changes in β-cell function have generally been ascribed to the rise in cyclic AMP levels. It has been reported, however, that methylxanthines exert a direct inhibitory effect on Ca uptake by islet microsomal fractions (Sehlin, 1976) or mitochondria (Sugden & Ashcroft, 1978) and it was occasionally suggested that their effects on insulin release were not necessarily mediated by cyclic AMP (Capito & Hedeskov, 1974; Henquin, 1978).

This study shows that many, but not all, changes in β-cell membrane potential brought about by low concentrations of theophylline are qualitatively similar to those produced by dibutyryl cyclic AMP. On the other hand, 10 mM-theophylline produced effects markedly different from those of the nucleotide. In contrast to 10 mM-theophylline (or 1 mM-isobutylmethylxanthine), dibutyryl cyclic AMP failed to depolarize the β-cell membrane in the presence of a non-stimulatory concentration of glucose. This depolarization is likely due to a direct decrease in K permeability of the β-cell membrane, as both methylxanthines, but not dibutyryl cyclic AMP, decreased $^{86}Rb^{+}$ efflux from rat islets under similar conditions (Henquin, 1980). Furthermore, the observation that only theophylline and isobutylmethylxanthine stimulated insulin release is in keeping with the report (Gylfe & Hellman, 1981) that in the absence of glucose 1 mM-isobutylmethylxanthine, but not 2 mM-dibutyryl cyclic AMP, accelerated $^{45}Ca^{2+}$ efflux from mouse islets. It is also remarkable that dibutyryl cyclic AMP and 10 mM-theophylline increased insulin release approximately to the same extent, but produced very different changes in electrical activity in the presence of 10 mM-glucose. The divergence was even more striking in the presence of 7 mM-glucose, when dibutyryl cyclic AMP triggered electrical activity (Henquin & Meissner, 1983), whereas 10 mM-theophylline hyperpolarized the membrane, although their secretory effects were quite similar.

The obvious implication of these observations is that changes in β-cell function (e.g. insulin release) do not necessarily result from the rise in cyclic AMP levels if the concentration of methylxanthine is high. Dibutyryl cyclic AMP and stimulators of the adenylate cyclase should be preferred to delineate the exact role of cyclic AMP in the stimulus–secretion coupling.

Mechanisms of the changes in electrical activity produced by dibutyryl cyclic AMP and theophylline

The increase in insulin release caused by dibutyryl cyclic AMP or theophylline in the presence of threshold or stimulatory concentrations of glucose is not thought to involve changes in the ionic permeabilities of the β-cell membrane, but to result from a direct or cyclic AMP-mediated translocation of Ca^{2+} from intracellular stores (Wollheim & Sharp, 1981). If this hypothesis were correct, no change in membrane potential would be expected, apart from an eventual hyperpolarization, caused by activation of Ca-sensitive K channels (Henquin, 1979; Atwater, Dawson, Ribalet & Rojas, 1979). Such a hyperpolarization was indeed observed upon addition of 2 or 10 mM-theophylline, and the rapid acceleration of $^{86}Rb^+$ efflux that occurs under these conditions (Henquin, 1980; Carpinelli & Malaisse, 1981) suggests that it is really due to an increase in membrane K permeability. Dibutyryl cyclic AMP which did not produce that initial hyperpolarization increased the rate of $^{86}Rb^+$ efflux much more slowly (Henquin & Meissner, 1983). On the other hand, both the nucleotide and the methylxanthine produced other changes in glucose-induced electrical activity that cannot be explained by a mere rise in cellular Ca^{2+}, but suggest hitherto unrecognized effects on the plasma membrane.

Dibutyryl cyclic AMP and the low concentrations of theophylline (0·5–2 mM) increased the duration of the slow waves and decreased the slope of their repolarization phase; they also decreased the duration of the silent intervals and increased the slope of the prepotential. These changes are compatible with an increase in Ca conductance and/or a decrease in K conductance (Atwater, Ribalet & Rojas, 1978; Atwater *et al.* 1979; Henquin, Meissner & Preissler, 1979; Meissner & Preissler, 1979; Meissner & Schmeer, 1981; Ribalet & Beigelman, 1980). Recent experiments with dibutyryl cyclic AMP provided support for the first possibility (Henquin & Meissner, 1983). On the other hand, the second possibility is in apparent conflict with the acceleration of $^{86}Rb^+$ efflux that occurs under these conditions (Henquin, 1980; Carpinelli & Malaisse, 1981). Furthermore, certain changes in the slow waves brought about by 10 mM-theophylline add to the complexity of the picture. Thus the duration of the slow waves was decreased and their repolarization phase accelerated, as if a K permeability was increased. Conversely, the slope of the prepotential was increased, as if another K permeabilty was decreased. Measurements of ionic fluxes in whole islets may not be sensitive enough to decide whether an increased Ca^{2+} influx results from a direct change in the properties of Ca channels or is secondary to subtle changes in K permeability. It is indeed possible that K channels with distinct properties (different sensitivities or responses to membrane potential, Ca^{2+} or cyclic AMP) mediate the repolarization of the spikes and of the slow waves, and the depolarization of the prepotential. An increase in K efflux through one type of channel could thus mask a relatively smaller decrease through channels of another type. It also seems pertinent to envisage that a rise in free cellular Ca^{2+} could inactivate the Ca conductance as it does in certain neurones (Eckert & Tillotson, 1981; Plant, Standen & Ward, 1983). Such a mechanism, which remains speculative for β-cells, could contribute to both the shortening of the slow waves by 10 mM-theophylline and the increase in activity ('off' response) observed upon its withdrawal.

Conclusions

The possibility that cyclic nucleotides may influence the ionic permeabilities of the plasma membrane through phosphorylation of certain proteins (Greengard, 1976) is receiving increasing attention. In many systems, oscillations in the membrane potential and in the cell function (contraction, secretion) appear to depend on a complex interplay between Ca^{2+}, cyclic AMP and the ionic permeabilities of the membrane (Berridge & Rapp, 1979). There exist striking analogies between the changes in membrane potential induced by cyclic AMP in β-cells and in at least two other tissues. In certain peptidergic neurones of *Aplysia*, the nucleotide decreases a K permeability and induces repetitive discharges of electrical activity (Strumwasser, Kaczmarek & Jennings, 1982), and in heart muscle cells cyclic AMP increases duration and frequency of the action potentials by a direct effect on Ca^{2+} channels (Reuter, 1983). On the other hand, cyclic AMP inhibits the spontaneous rhythmic electrical activity of the *Aplysia* neurone R 15 (Levitan & Adams, 1981) by increasing K permeability of the membrane. The present and two recent studies with dibutyryl cyclic AMP (Henquin & Meissner, 1983) and forskolin (Henquin, Schmeer & Meissner, 1983), an activator of the adenylate cyclase, strongly suggest that cyclic AMP also influences the ionic permeabilities of the plasma membrane in pancreatic β-cells. Further experiments will be necessary, however, to characterize these changes, to determine to what extent they control the oscillations in membrane potential recorded in the presence of physiological concentrations of glucose, and to establish precisely their role in the stimulus–secretion coupling.

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