K^+ Depolarization and Phospholipid Metabolism in Frog Sartorius Muscle

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Abstract. K^+ depolarization evokes phosphatidylinositol response, i.e. the increased ^{32}P orthophosphate labelling of phosphatidylinositol in frog sartorii muscles. The phosphatidylinositol response seems to be closely related to K^+ depolarization and not to the transient Ca^{2+} release at the beginning of depolarization. It ceases as soon as the muscles depolarized by 90 mmol/l KCl for a short period of time are repolarized, while it continues when the depolarization is maintained. When the muscles are depolarized with 20 mmol/l KCl, the phosphatidylinositol response is also observed. This response is not suppressed by drugs that block Ca^{2+} mobilization. Other agents like caffeine, azide or EGTA which induce some effects similar to that of K^+ depolarization, do not evoke phosphatidylinositol response. Rather, they simply cause a decrease in the labelling of phospholipids, phosphatidylinositol being the least affected. In muscles derived from frogs maintained under healthy conditions Ca^{2+} release in the early phase of K^+ depolarization does not cause significant changes in phospholipid labelling. However, in muscles from frogs starving for many months, a large decrease in the labelling of phospholipids is observed in the early phase of K^+ depolarization. It is postulated that the changes in the physicochemical state of the membrane and not Ca^{2+} gating mechanism or free cell Ca^{2+} level are crucial in the phosphatidylinositol response in the frog sartorii muscles depolarized by high K^+.

Key words: K^+ depolarization — ^{32}P phospholipid labelling — Frog sartorius muscle

Introduction

An increased turnover of tissue phosphatidylinositol in the stimulated state, the so called phosphatidylinositol effect or response, is observed in tissues when physiological agonists interact with some membrane receptors. This response is initiated by the hydrolysis of the lipid by phospholipase C to diacylglycerol and inositol phosphate. The underlying mechanism and physiological significance of the phosphatidylinositol response have been discussed by many authors in recent years (Michell 1979; Putney 1981; Hawthorne 1982; Michell 1982). In an earlier paper (Novotný et al. 1978) we have described the increased ^{32}P labelling of phos-

phatidylinositol during K^+ depolarization in frog sartorii muscles. This phosphatidylinositol response seems to differ from the phosphatidylinositol responses mentioned above. K^+ depolarization of frog sartorius is not accompanied by agonist-receptor interaction. It appears that a close connection between K^+ depolarization and the phosphatidylinositol response exists. Our earlier results have not excluded the possibility that the long-lasting increased labelling of phosphatidylinositol is a result of its splitting during the first minutes of K^+ depolarization, when the free Ca^{2+} level in myoplasm is high. We have therefore conducted other experiments to ascertain whether the increased ^{32}P labelling of phosphatidylinositol coincides directly with the period of depolarization. The results of some other experiments which argue against the involvement of Ca^{2+} ions mobilization in the phosphatidylinositol response are also reported.

Material and Methods

The procedures used in this study had been described previously (Novotný et al. 1978), therefore only a brief outline will be given in the present report.

The experiments were carried out on frog sartorius muscles weighing from 70 to 100 mg (*Rana temporaria*). After dissection the muscles were attached at resting length to glass rods covered with polyethylene tubing, with steel pins. Three paired muscles were attached to each glass rod. One group of paired muscles served as a control. The muscles were being kept for 30 min in oxygenated Ringer solution containing (in mmol/l) NaCl 111, KCl 3, $CaCl_2$ 1, Na_2HPO_4 2,15, NaH_2PO_4 0.85, pH 7.2. They where then transferred into vessels containing 7 ml of Ringer solution with 18.5 MBq of ^{32}P orthophosphate (carrier-free; from Isocommerz GmbH Berlin) or with 3.7 MBq (2-^3H) glycerol (370 Gbq/mol, from Amersham). Oxygen was bubbled through the solution, and the temperature was maintained at 22 °C. For the first two hours both control and experimental muscles were kept in Ringer solution containing ^{32}P orthophosphate or ^3H glycerol. After two hours the experimental muscles were treated as specified in the text. After incubation the muscles were frozen in liquid nitrogen. Lipids were extracted and separated by thin-layer chromatography as previously described. The results are expressed as counts. $min^{-1}.\mu mol^{-1}$ total lipid P or as percentage of control values. The data are the means ±S.E.M.

Results

K^+ depolarization and ^{32}P labelling of phospholipids. The data presentend in Fig. 1 show that K^+ depolarization causes an increase in the labelling of phosphatidylinositol, and that this increase is not a consequence of splitting of phosphatidylinositol in the early phase of K^+ depolarization when the level of free intracellular calcium is transiently increased. When paired muscles were kept for 30 min in 90 mmol/l KCl Ringer solution and then for the next 90 min either in high KCl Ringer solution or in normal (3 mmol/l KCl) Ringer solution, only muscles in high KCl Ringer solution continued to incorporate ^{32}P orthophosphate at an increased rate. Since, in our earlier experiments (Novotný et al. 1978) we did not find any significant difference in ^{32}P labelling of phosphate precursors of

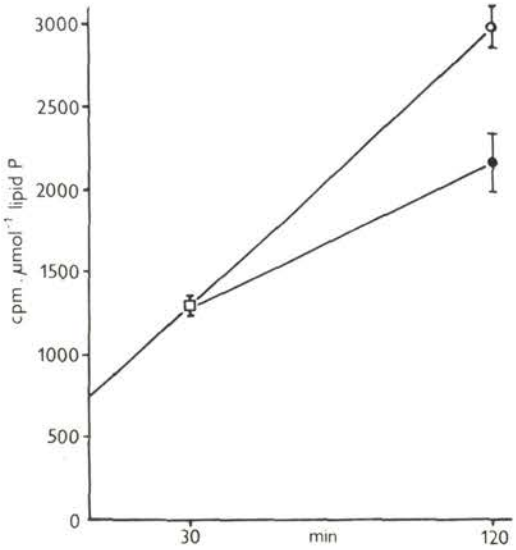

Fig. 1. The effect of repolarization on the incorporation of ^{32}P orthophosphate into phosphatidylinositol of muscle depolarized by K^+ ions.
Square — 30 min 90 mmol/l KCl Ringer solution; solid circle — 30 min 90 mmol/l KCl Ringer solution plus 90 min 3 mmol/l KCl Ringer solution; open circle — 120 min 90 mmol/l KCl Ringer solution. All solutions with ^{32}P orthophosphate. Prior to K^+ depolarization the muscles were incubated for 2 hours in radioactive Ringer solution as described in the Methods section.

phospholipids between muscles in normal Ringer solution and in high KCl Ringer solution, we ascribe the return of ^{32}P labelling of phosphatidylinositol to the return of phosphatidylinositol phosphodiesterase (phospholipase C) activity to the level which it had before depolarization.

The close connection between K^+ depolarization and phosphatidylinositol effect has further been indicated by experiments in which the muscles were depolarized with 20 mmol/l KCl and drugs blocking Ca^{2+} mobilization applied. In 20 mmol/l K^+ Ringer solution the increase in the free cell Ca^{2+} level is small and in contrast to the depolarization induced by 90 mmol/l KCl lasting for the whole period of depolarization (Bianchi and Shanes 1959; Novotný 1965). In muscles incubated for two hours in 20 mmol/l KCl Ringer solution the phosphatidylinositol response is smaller than that observed in 90 mmol/l KCl (Novotný et al. 1978), the labelling of phosphatidylcholine and phosphatidylethanolamine being unaffected. The increase in the free Ca^{2+} level induced by 20 mmol KCl can be completely suppressed by procaine or phenobarbital (Novotný and Vyskočil 1963, 1966) without any effect on the level of K^+ depolarization. However, the increased ^{32}P

Table 1. Effects of depolarization by 20 mmol/l KCl on ^{32}P labelling of phospholipids of frog sartorii.

	^{32}P incorporation (% of control)		
	PC	PE	PI
20 mmol/l K^+ Ringer	99 ± 10	108 ± 11	138 ± 9^{++}
20 mmol/l K^+ Ringer plus 1 mmol/l procaine	111 ± 11	101 ± 12	141 ± 10^{++}
20 mmol/l K^+ Ringer plus 0,1 mmol/l phenobarbital	108 ± 8	102 ± 9	134 ± 8^{11+}

The muscles were incubated for 2 h in radioactive Ringer solution prior to K^+ depolarization. The duration of K^+ depolarization was 2 h (radioactive solution).
For further details see Methods section.
The values represent mean ± S.E.M. $++P<0,01$; $+0,01<P>0,05$; all by Student's t- test. The data are averages from 8—12 experiments.
PC — phosphatidylcholine, PE — phosphatidylethanolamine, PI — phosphatidylinositol.

labelling of phosphatidylinositol is suppressed neither by procaine nor by phenobarbital (Table 1).

It was of interest to ascertain whether there might be some change in radioactivity of previously labelled phospholipids in the early phase of depolarization by 90 mmol/l KCl when the level of free Ca^{2+} is greatly increased and the muscle is in a state of contracture for a few minutes. When the muscles were exposed to 90 mmol/l KCl Ringer solution for 5 min, no significant change in phospholipid content or in the radioactivity of phosphatidylinositol and phosphatidylcholine (determined per wet weight of the tissue) was found (105.7 + 18.7 % of control for phosphatidylinositol and 102.7 + 26 % of control for phosphatidylcholine). It seems therefore that during the massive mobilization of Ca^{2+} ions in the twitch muscle fibres the phospholipases C are not activated by Ca^{2+} to an extent which would cause a measurable decrease in membrane phospholipids.

Other effects on ^{32}P labelling of phospholipids. Certain conditions inducing some of the effects in muscle fibres which occur during K^+ depolarization, i. e. rise in free Ca^{2+} level or depolarization, did not result in an increase in phosphatidylinositol labelling (Table 2). Caffeine, at a concentration which only slightly increases free cell Ca^{2+} level (Bianchi 1961) and causes no contracture, does not significantly change phospholipid labelling. 4 mmol/l caffeine which induces a large increase in free Ca^{2+} levels, depletion of energy stores and contractures, decreases ^{32}P labelling of all phospholipids, phosphatidylinositol being the least affected. The decrease in phospholipid labelling by 4 mmol/l caffeine can be ascribed to a decrease in energy stores. A similar decrease in phospholipid labelling was found when the muscles were incubated with 2 mmol/l azide. 2 mmol/l azide causes a decrease of membrane potential from 92 mV to 65 mV (F. Vyskočil, unpublished data),

Table 2. Various influences affecting ^{32}P labelling of phospholipids of frog sartorii.

		^{32}P incorporation (% of control)		
		PC	PE	PI
caffeine	2 mmol/l	93 ± 11	109 ± 9	98 ± 11
	4 mmol/l	38 ± 4	42 ± 7	59 ± 3
azide	1 mmol/l	95 ± 9	98 ± 9	101 ± 6
	2 mmol/l	36 ± 5	45 ± 6	67 ± 4
EGTA	4 mmol/l	66 ± 7	85 ± 9	100 ± 86

As described for Table 1 the muscles were incubated for 2 h prior to the experimental treatment (2 h radioactive solution). The data are averages from 8 to 10 experiments.
PC — phosphatidylcholine, PE — phosphatidylethanolamine, PI — phosphatidylinositol.
EGTA — in 0 Ca^{2+} Ringer solution.

however, in this case depolarization is accompanied by a suppression of cytochrome oxidase activity and depletion of energy stores (Stannard 1939; Briner et al. 1959).

The depolarization of the frog sartorius can be further achieved by removing Ca^{2+} free Ringer solution (Pauschinger et al. 1964). In Ca^{2+} free Ringer solution plus 4 mmol/l EGTA the membrane potential decreases during the first 60 min. from 94 mV to 68 mV and in the next 60 min. to 64 mV (F. Vyskočil, unpublished data). Under these conditions after two hours of incubation we found a decrease in ^{32}P labelling of phosphatidylcholine and phosphatidylethanolamine and no change in labelling of phosphatidylinositol. The effect of removing Ca^{2+} is not quite clear. Nevertheless, it seems evident that depolarization by removing Ca^{2+} does not evoke a phosphatidylinositol response similar to that of K^+ depolarization.

It has been shown that phosphatidylinositol- phosphodiesterase was specifically stimulated by oleic acid when membraneous phosphatidylinositol substrate was used (Irwine et al. 1979b). We tested the effect of 0.1 mmol/l oleic acid, sonicated in Ringer solution, but did not observe any effect.

K^+ depolarization and ^3H glycerol labelling of phospholipids.

Attempts were made to find out whether the changes in ^{32}P orthophosphate labelling are paralleled by changes in ^3H glycerol labelling. Unfortunately, the incorporation of ^3H glycerol tended to level off after 2 hours of incubation with a large variability in individual measurements so that it was not possible to measure minor changes in ^3H glycerol labelling. Nevertheless, some of the major changes that we observed are worthy of reporting.

A large increase in intracellular Ca^{2+} during the early phase of K^+ depolarization by 90 mmol/l KCl did not cause any measurable changes in membrane phospholipids. If, however, the effect of K^+ depolarization was measured in muscles from frogs, which had starved for many months in the Winter and Spring until June, a large decrease in the radioactivity of ^3H glycerol prelabelled

phospholipids was found. After 10 min of exposure to 90 mmol/l KCl Ringer solution, the radioactivity of previously ^{3}H glycerol labelled phospholipids, determined per wet weight of the tissue, decreased in the case of phosphatidylcholine to 38 ± 6 %, phosphatidylethanolamine to 25 ± 6 % and phosphatidylinositol to 34 ± 8 % (4 exp.) as compared to the control muscles for which the labelling proceeded at a rate similar to that of healthy frogs. The decline in ^{3}H glycerol labelling in muscles from frogs after long-lasting starvation brought about by short exposure to high KCl obviously means that phospholipids in these muscles are exposed to a much larger extent to phospholipases during depolarization than phospholipids in muscle derived from frogs maintained under healthy conditions. A low level of ATP in muscle cells after long-lasting starvation may play a role in these effects, since it appears that ATP protects membrane phospholipids from the attack by phospholipases (Gazzitt et al. 1975; Higgins et al. 1981).

Discussion

The data presented in this paper support our earlier view (Novotný et al. 1978), that it is K^+ depolarization and not Ca^{2+} mobilization that is responsible for the increase in ^{32}P orthophosphate labelling of phosphatidylinositol and phosphatidylserine in the frog sartorius muscle. Two observations are in favour of our assertion: firstly, the increased ^{32}P labelling of phosphatidylinositol ceases when the muscle is repolarized after short period of K^+ depolarization and consequently the long lasting increased labelling cannot be related to the transient Ca^{2+} mobilization in the early phase of K^+ depolarization; secondly, the depolarization with 20 mmol/l KCl increases labelling of phosphatidylinositol in the presence of drugs inhibiting Ca^{2+} mobilization. It is justifiable, therefore to suppose that it is not the level of free Ca^{2+} which controls phosphatidylinositol-phosphodiesterase (phospholipase C) activity in muscle depolarized by potassium ions. Similarly Irwine et al. (1979a) state that the free Ca^{2+} level in the cell is unlikely to be a universal activator of the enzyme in vivo. According to Irwine et al. (1979a) sufficient Ca^{2+} is bound to the cellular membranes of liver and brain to satisfy the enzyme's requirement. The observation made by Irwine et al. (1979a) indicates that the activity of cytosolic phosphodiesterase may be controlled primarily by the physicochemical state of the membrane substrate since the hydrolysis of phosphatidylinositol by Ca^{2+} -dependent cytosolic phosphatidylinositol-phosphodiesterase from rat brain is extremely slow, when the membrane hydrolyses phosphatidylinositol integrated in membrane, in comparison with hydrolysis of pure phospholipid. According to Irwine and coworkers (1979a) such suppression can be removed by physico-chemical changes in the membrane such as phase separations caused either by changes in protein conformation or ion concentration.

As the conformational state of membrane proteins and phospholipids is closely related to the local potential of charged groups in excitable membranes (Strickholm 1981), it seems quite reasonable to assume that K^+ depolarization may be the primary cause of the phosphatidylinositol response in frog sartorii.

The question still remains, however, whether it is the depolarization itself or the high K^+ion concentration which evokes the phosphatidylinositol response. This question is difficult to answer. It has been stated by Strickholm that a depolarization induced by raising external K^+ does not necessarily results in the same changes in excitable membranes as those induced by an equivalent depolarization e.g. by voltage clamp in steady state. These differences are not clear although there are physico-chemical reasons for believing that not only the membrane potential but also the local ionic environment may be important factors in determining the conformation state of membrane proteins and lipids.

The phosphatidylinositol response to K^+ depolarization appears to be rather specific, since conditions which in some way induce changes in the frog sartorii similar to those induced by high K^+ (a decrease in membrane potential, an increase in the free Ca^{2+} level), do not result in an increase in ^{32}P labelling of phosphatidylinositol. The effects of azid accompanied by a decrease in membrane potential or caffeine with a resulting increase in the free cellular Ca^{2+} level do not bring about an increase in ^{32}P labelling of phosphatidylinositol, on the contrary, they lead to a decrease of the phosphatidylinositol labelling. Similarly, in Ca^{2+} free Ringer solution where muscles are slightly depolarized, the phosphatidylinositol response is not observed. Most of these effects reported in Table 2 bring about a decrease in the labelling of phospholipids, phosphatidylinositol being the least affected. These findings seem to be of some importance from two aspects.

1. The experiments with caffeine show that the changes in glycolytic intermediates and other phosphates cannot play any role in the phosphatidylinositol response observed in 20 mmol/l K^+ Ringer solution. Otherwise, 2 mmol/l caffeine, which induces changes in glycolysis and respiration similar to those induced by 20 mmol/l K^+, should also induce the phosphatidylinositol response.
2. As the ^{32}P labelling of phosphatidylinositol is depressed to a lesser extent than the labelling of other phospholipids, processes regulating the synthesis of phosphatidylinositol seem to differ from those regulating the synthesis of phosphatidylcholine and phosphatidylethanolamine. Certainly, there are differences in metabolic pathways of these three phospholipids: only phosphatidylinostitol is synthetized from phosphatidic acid. Nevertheless, it seems that some preference for the synthesis of phosphatidylinositol also exists. There is no explanation at present for these differences also observed by other authors (Strunecká et al. 1978). Hypothetically, it might be supposed that cytidintriphosphate would be preferentially used for the synthesis of phosphatidylinositol, if this nucleotide is a universal

signal for the biosynthesis of all phospholipids as suggested by Vance and Choy (1979).

In summary it is apparent from the results presented in this and in the former paper (Novotný et al. 1978) that the stimulated turnover of phosphatidylinositol in the frog sartorius muscle depolarized with high KCl and consequently phosphatidylinositol phosphodiesterase activity which is of crucial significance in the turnover of phosphatidylinositol, are not controlled by processes related to Ca^{2+} gating mechanism or to the free Ca^{2+} level in the muscle fibres. The exact way how phosphatidylinositol phosphodiesterase activity is modified in depolarized muscle has yet to be explored, similarly as the physiological significance of this change.Since the phosphatidylinositol response in depolarized twitch muscle fibres appears to be rather late phenomenon following the transient Ca^{2+} release the processes maintaining the Ca^{2+} system in the inactive state might be looked after for physiological explanation.

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