KR

PICRYLACETATE AN INHIBITOR FOR Ca^{2+}-PHOSPHATE SYMPORT

R. Kiehl

Institut für Physiologische Chemie der Ruhr-Universität

Bochum, 4630 Bochum, Universitätsstraße 150, FRG

27. Aug. 1980, FEBS Letters, subm.

1. Introduction

Picrylacetate (PA), 250 µM, has been reported to uncouple oxidative phosphorylation and inhibit partly respiration in rat liver mitochondria [1]. This effect has been ascribed to picric acid liberated inside the mitochondria. Picric acid as such has no effect on mitochondria [1,2] but in submitochondrial particles it inhibits oxidative phosphorylation causing only a small increase in proton permeability if compared with DNP [2].

From the chemist's point of view, PA is a highly reactive acetylating reagent rather than a picrate-generating system. For this reason the effect of the compound on mitochondria was reinvestigated. A preliminary communication of some of the results has been presented [3].

2. Materials and methods

Rat liver mitochondria were prepared essentially according to [4], submitochondrial particles to [5]. Protein was estimated by the biuret method [6]. Oxygen uptake at 30°C was measured polarographically, ATPase spectrophotometrically at 30°C [7]. O_2-pulse experiments with O_2-saturated 0,25 M sucrose at 30°C were done essentially according to [8] with the equipment described in [2].

O-acetyl-picric acid was prepared as in [1]. All other reagents used were of the highest commercial purity available.

Abbreviations: PA, picrylacetate; CAT, carboxyatractyloside; Oli, oligomycin; DTE, dithioerythritol; DNP, 2,4-dinitrophenol; CCCP, carbonyl-cyanide m-chloro-phenylhydrazone; Rot, rotenone; PMB, p-mercuribenzoate; BSA, bovine serum albumine; NPTU, N-phenyl-N'-

n-nonylthiourea; NSPM, N'-[N"-n-nonyl-4-sulfamoylphenyl]-maleimide; DH, dehydrogenase.

3. Results

30 to 35 nmol PA/mg protein inhibits state 4 → state 3 transition of rat liver mitochondria at sites I - III (13 to 15 μM) and II - III (3 to 5 μM; Fig. 1,3) to more than 90%. Picric acid as such has no effect at higher concentrations. There is always about 10% PA insensitive state 4 → state 3 transition.

Inhibition of state 4 → state 3 transition with ß-hydroxybutyrate as substrate cannot be observed because of the concomitant inhibition of respiration at 6 to 10 nmol PA/mg protein (3 to 5 μM).

The effects on oxidative phosphorylation and respiratory activity are not reversed by addition of BSA. However, a slight stimulation of state 4 by PA is reversed by BSA-treatment.

The inhibition of ADP induced state 4 → state 3 transition is released by uncouplers to about state 3 rate and reversed by DTE (15 mM) to about 50%. Note the state 3 → state 4 transition at addition of DTE after PA blocking of ADP induced transition (Fig. 1).

In the presence of 30 to 40 μM PA the mitochondrial respiration rate is below state 4 level and cannot be increased by uncouplers.

Arsenate (1.7 mM) and Ca^{2+} (1.7 mM)-stimulated succinate oxidase activities are inhibited by 30 to 35 nmol PA/mg protein, which therefore acts differently than the inhibitors CAT or oligomycin (Fig.1,2). See ref. [9] for effect of atractylate and oligomycin on arsenate stimulated respiration. Two other findings in Fig. 1,2 are of interest: 1. DTE (17 mM) added to the various inhibitor/arsenate treated particles (Fig. 1) stimulates respiration to some extent. 2. At higher arsenate concentration (2.8 mM) none of the above inhibitors are able to prevent completely arsenate stimulation and DTE seems to stimulate even more (Fig. 2).

0.57 mM Ca^{2+}-ions in presence of 10 mM succinate induce a rapid oxygen uptake, which lasts until oxygen is exhausted if enough phosphate is present. The minimal concentration of phosphate is about 1.2 mM. In the presence of smaller concentrations such as 0.6 mM phosphate the increased respiratory activity declines after a certain time. It can be restored by addition of more phosphate. Similar results were obtained for arsenate (Fig. 3). The rat liver mitochondria used for the studies therefore have a large capacity to accumulate Ca^{2+} and phosphate on succinate oxidation. This is not true for site I - III respiration, where fast inhibition of respiration takes place on massive Ca^{2+} and phosphate loads; see also [10,11].

The addition of 30 to 35 nmol PA/mg protein shortly (about 2 sec) after addition of Ca^{2+} in the presence of phosphate (or arsenate; >1.2 mM) inhibits the increased respiration rate after a lag of about 15 seconds. After further 50 seconds the respiration starts to increase again to about 35% of the initial Ca^{2+} induced respiratory rate. The lag of 50 seconds can be abolished by addition of uncouplers at the inhibited state (Fig. 4). DTE (25 mM) is not able to release the inhibition by PA. CAT and oligomycin added together do not have any effect on the PA inhibition of the Ca^{2+}/phosphate or Ca^{2+}/arsenate accumulation (Fig. 4) nor on the accumulation itself.

The ATPase of whole mitochondria is stimulated with 40 μM PA to a maximum of 5 times the original ATPase. (0.1 μM ATP/min mg) (Fig.5).

Oxygen pulse experiments on whole mitochondria (succinate as substrate) show that a maximum of inhibition of $t_{1/2}$ of H^+-reentry (by a factor of 1.4 to 1.5) is obtained with 40 to 50 μM PA. At 150 μM PA there is a time dependent stimulation of H^+-reentry by a factor of 6 to 7 (Fig. 5). Stimulation of ATPase and inhibition of $t_{1/2}$ are parallel effects and occur at about the same μM concentrations of PA which keep respiration below state 4 level (at state 6 level).

In the presence of submitochondrial particles PA is hydrolyzed rapidly, and studies as done on mitochondria are not possible. However, at high PA concentration (about 100 μM), where similar concentrations of picric acid stimulate ATPase, the ATPase activity is also increased. There is about 80% inhibition of ATPase at about 40 μM immediately after addition of PA. After 4 minutes incubation with PA the inhibition is only about 50% (50% inhibition of ATP-P_i-exchange is at about 7-10 μM PA). A similar result is also obtained with the new uncoupler NPTU.[1)]

4. Discussion

The lipophilic picrylacetate exerts several effects on mitochondrial functions. The most sensitive enzyme toward PA tested so far in the mitochondrial inner membrane is the ß-hydroxybutyrate dehydrogenase, which is blocked at 6 to 10 nmol PA/mg protein. The DH is also very sensitive toward other lipophilic reagents: it is inhibited and labeled very rapidly in a Diamide sensitive way by the lipophilic thiol reagent NSPM [12] indicating involvement of at least two vicinal thiols in the active center of the enzym. It is possible that these thiol groups are acetylated by PA, but reactions with other nucleophilic groups are of course possible.

The inhibition of state 4 → state 3 transition by 30 to 35 nmol PA/mg protein has been shown to be different from the effects of CAT or oligomycin (arsenate, Ca^{2+}experiments) and to be due to reversible chemical modification (BSA or DTE treatment). Reaction on the phosphate-translocator is most likely. Since arsenate is translocated via the phosphate-translocator [13,14] and interacting with the ATPase [15,16], also affecting at least two enzyme systems, the concentration of arsenate is important for the differentation of the

---

1) R. Kiehl and Y. Hatefi, unpublished experiments.

PA, CAT or oligomycin effects. The inhibition of state 4 → state 3 transition by PA is in some aspects similar to the effect of Diamide on state 4 → state 3 transition [17].

That PA is reacting on the phosphate translocator can be implicated by the effect of PA on the succinate driven Ca^{2+}-accumulation catalyzed by phosphate or arsenate (at a PA concentration which gave 80 - 90% inhibition of state 4 → state 3). In this system PA mimicks lack of phosphate or arsenate and inhibits Ca^{2+}-accumulation. The time dependence suggest also (as BSA treatment does) that covalent modification by PA is taking place. DTE is not able to release the inhibition by PA although it is effective in PA inhibited ADP induced state 4 → state 3 transition. The only difference between these systems is Ca^{2+}. Uncouplers are still able to stimulate to some extent respiration in the system were succinate oxidation stimulated by Ca^{2+}/phosphate-accumulation is inhibited. Even in the absence of uncouplers a delayed stimulation of respiration takes place, presumably by liberated picric acid during PA reaction with nucleophilic groups. As expected oligomycin and CAT do not have any effect on Ca^{2+}/phosphate-accumulation nor on its inhibition by PA. PA is as inhibitor very similar to PMB [18].

The experiments show also that there is no energy linked Ca^{2+}-accumulation if phosphate is missing (even in the presence of succinate), and that the ratio of phosphate and Ca^{2+}-concentration is about 2 [19].

From the results shown (massive loading experiments with Ca^{2+}), I like to bring into discussion a Ca^{2+}-phosphate symport by a multisubunit enzyme (a Ca^{2+}-phosphate symport system has already been described by Mitchell [20,21] and also a multisubunit enzyme for Ca^{2+} translocation [22,23]), which is in my opinion better able

to explain the results with PA as two independent carriers. Then picrylacetate should acetylate nucleophilic groups which are not only involved in translocation of phosphate but also of Ca^{2+}.

The effects at 40 µM PA are more complex and are involving at least two different interactions with the ATP-synthetase-complex (studies on ATP-P_i-exchange and ATPase of submitochondrial particles are supporting this conclusion). The first appears to be an acetylation of proteins in the complex with concomitant inhibition of the H^+-translocation (Fig. 5). In addition, the liberated picric acid should be able to bind at the uncoupler-binding site [2].

The effect of the compound on submitochondrial particles and isolated oligomycin-sensitive ATPase complex are presently under investigation.

Analyses of preliminary labeling experiments with ^{3}H-PA under conditions by SDS-gel electrophorese where labile acetyl-groups (^{3}H-acetyl groups) are expected to be stable (no mercaptoethanol, pH 5) showed labeling of polypeptides at M_r 30 000,10 000 and small M_r compounds (lipids etc.). Therefore as expected most of the effects by PA can be explained by the acetylation of proteins. The relative specific effects of PA as acetylating reagent may be due to the charge transfer interactions of the picryl residue with aromatic side chains.

Acknowledgement

I thank Dr. W.G. Hanstein for discussion and help in preparing this manuscript.

References

[1] Leader, J.E. and Whitehouse, M.W. (1966) Biochem. Pharmacology 15, 1379 - 1387

[2] Hanstein, W.G. and Hatefi. Y. (1974) Proc. Nat. Acad. Sci. USA 71, 288 - 292

[3] Kiehl, R. (1980) 1°EBEC Reports Vol., 295 - 296

[4] Kaschnitz, R.M., Hatefi, Y. and Morris, H.P. (1976) Biochim. Biophys. Acta 449, 224 - 235

[5] Löw, H. and Vallin, L. (1963) Biochim. Biophys. Acta 69, 361 - 374

[6] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751 - 766

[7] Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322 - 3329

[8] Archbold, G.P.R., Farington, C.L., Lappin, S.A., McKay, A.M. and Malpress, F.H. (1979) Biochem. J. 180, 161 - 174

[9] Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 707 - 716

[10] Lehninger, A.L., Vercesi, A. and Bababunmi, E.A. (1978) Proc. Nat. Acad. Sci. USA 75 1690 - 1694

[11] Vercesi, A., Reynafarje, B. and Lehninger, A.L. (1978) J. Biol. Chem. 253, 6379 - 6385

[12] Kiehl, R. (1980) 1°EBEC Reports Vol., 113 - 114

[13] Chappell, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 393 - 402

[14] Tyler, D.D. (1969) Biochem. J. 111, 665 - 678

[15] Estabrook, R.W. (1961) Biochem. Biophys. Res. Commun. 4, 89 - 91

[16] Kayalar, C., Rosing, J. and Boyer, P.D. (1977) J. Biol. Chem. 252, 2486 - 2491

[17] Siliprandi, D., Scutari, G., Zoccarato, F., Siliprandi, N. (1974) FEBS Letters 42, 197 - 199

[18] Fonyo, A. and Bessman, S.P. (1968) Biochem. Med. 2, 145 - 163

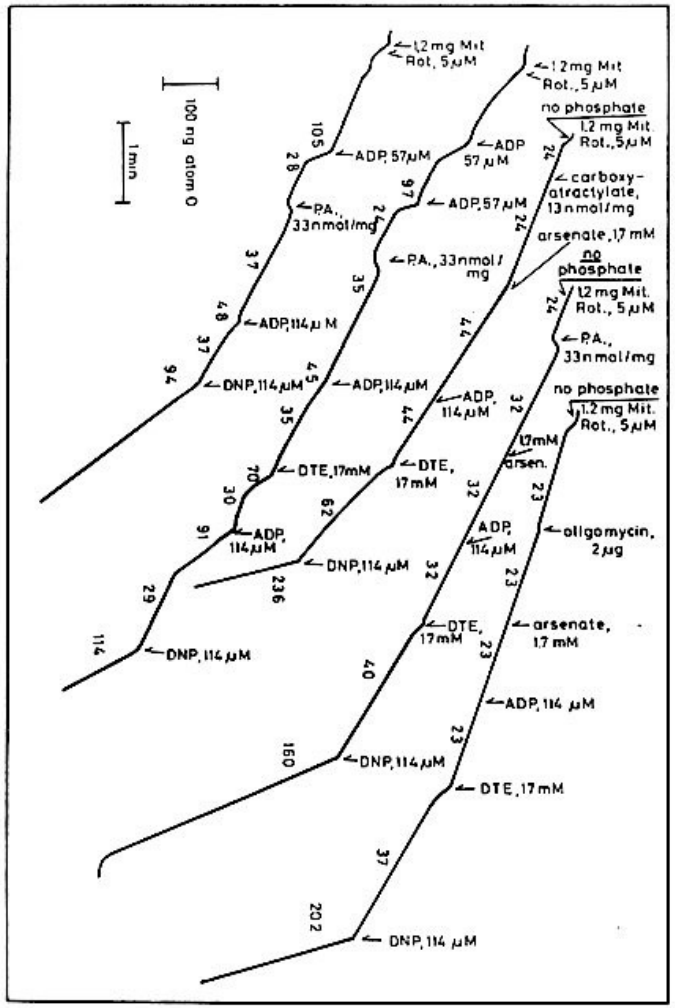
[19] Lehninger, A.L., Reynafarje, B., Vercesi, A., Few, W.P. (1978) Ann. of the N.Y.Acad. of Sciences 307, 160 - 176

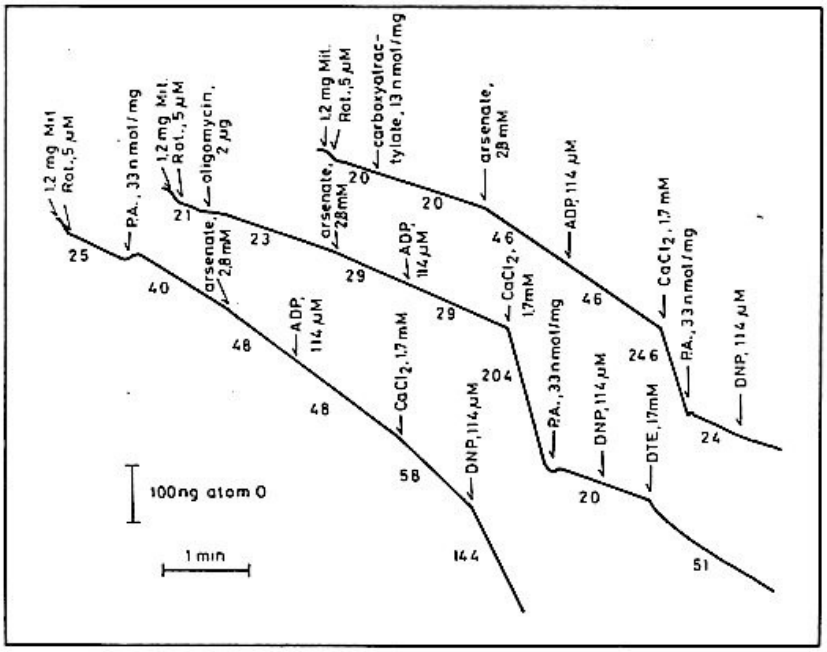
[20] Moyle, J. and Mitchell, P. (1977) FEBS Letters 73, 131 - 136

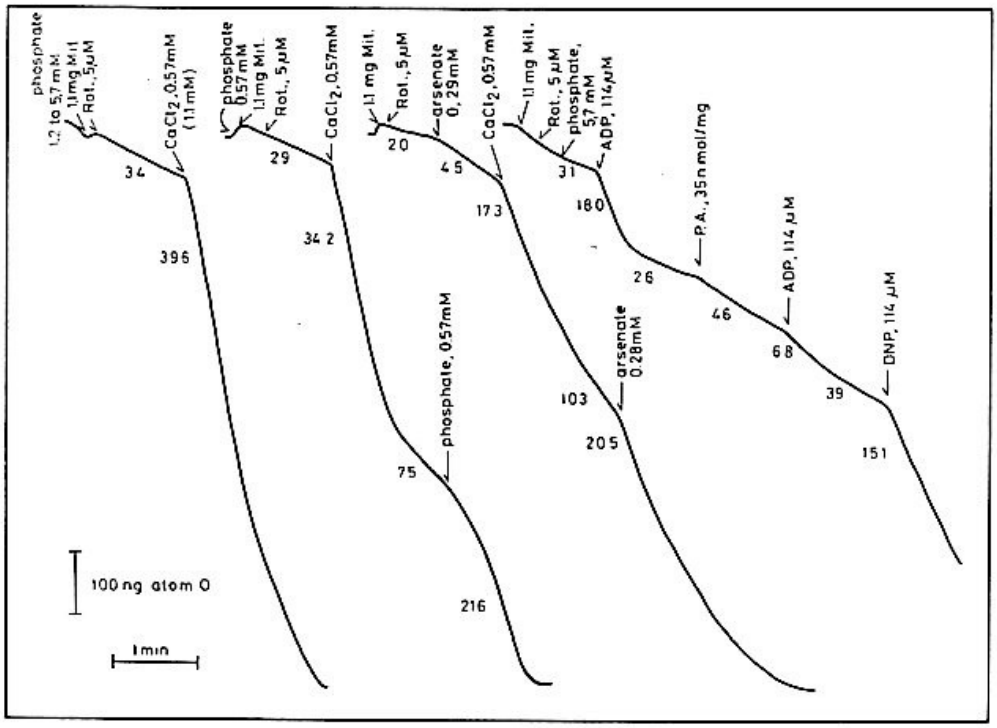
[21] Moyle, J. and Mitchell, P. (1977) FEBS Letters 77, 136 - 140

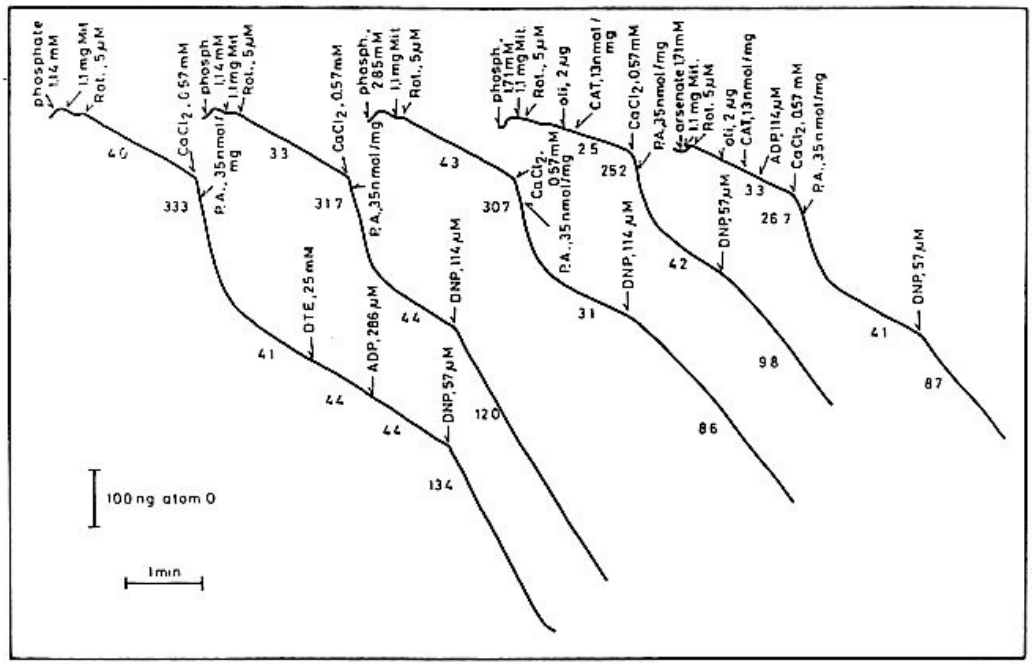
[22] Bragadin, M., Pozzan, T. and Azzone, G.F. (1979) Biochemistry 18, 5972 - 5978

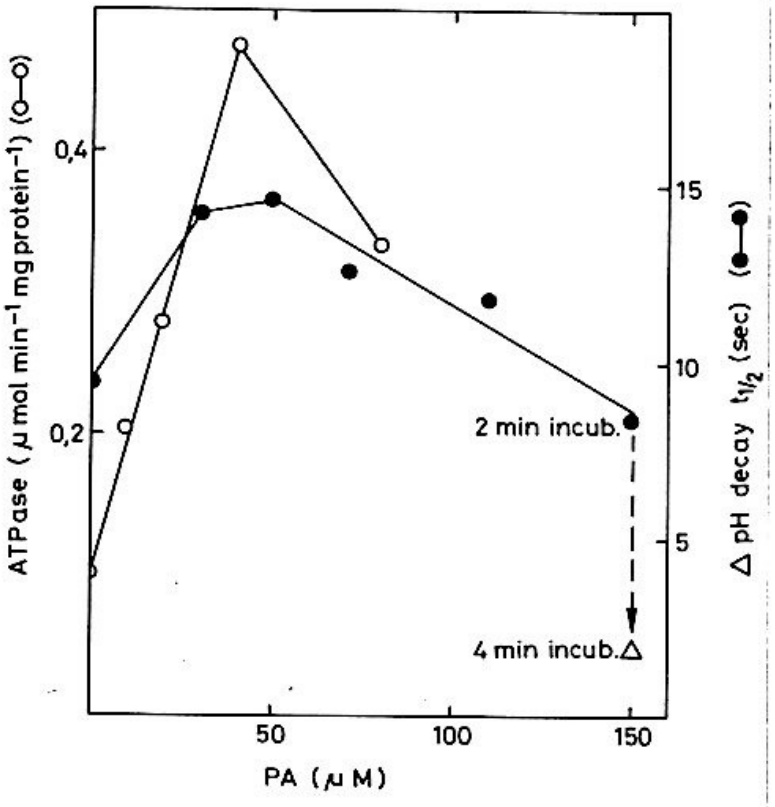
[23] Reed, K.C. and Bygrave, F.L. (1975) Eur. J. Biochem. 55, 497 - 504


100 ng atom O
1 min
1.2 mg Mit
Rot., 5 μM
ADP, 57 μM
P.A., 33 nmol/mg
ADP, 114 μM
DNP, 114 μM
DTE, 1.7 mM
no phosphate
carboxy-atractylate, 1.3 nmol/mg
arsenate, 1.7 mM
oligomycin, 2 μg
105
28
37
48
94
45
35
70
30
91
29
114
97
2
35
44
62
236
24
24
27
32
44
32
23
32
23
23
40
23
160
37
202


1.2 mg Mit
Rot., 5 µM
PA., 33 n mol/mg
1.2 mg Mit.
Rot., 5 µM
oligomycin, 2 µg
arsenate, 2.8 mM
1.2 mg Mit.
Rot., 5 µM
carboxyatractylate, 13 n mol/mg
arsenate, 2.8 mM
ADP, 114 µM
CaCl2, 1.7 mM
PA., 33 n mol/mg
DNP, 114 µM
DTE, 17 mM
CaCl2, 1.7 mM
PA., 33 n mol/mg
DNP, 114 µM
25
40
21
23
48
20
29
20
46
29
204
46
246
24
48
58
144
20
51
100ng atom O
1 min


phosphate
1.2 to 5.7 mM
1.1mg Mit
Rot., 5μM
CaCl2, 0.57mM
(1.1mM)
34
396
phosphate
0.57mM
1.1mg Mit.
Rot., 5μM
29
CaCl2, 0.57mM
342
phosphate, 0.57mM
75
216
1.1 mg Mit.
Rot., 5μM
20
arsenate
0, 29mM
45
CaCl2, 0.57 mM
173
arsenate
0.28mM
103
205
1.1 mg Mit.
Rot., 5 μM
phosphate,
5.7 mM
ADP, 114μM
31
180
P.A., 35 nmol/mg
26
ADP, 114 μM
46
DNP, 114 μM
68
39
151
100 ng atom O
1 min


phosphate 1.14 mM
1.1 mg Mit
Rot., 5 µM
40
333
CaCl2, 0.57 mM
phosph 1.14 mM
1.1 mg Mit
Rot., 5 µM
P.A., 35 nmol/mg
33
317
CaCl2, 0.57 mM
phosph., 2.85 mM
1.1 mg Mit
Rot., 5 µM
P.A., 35 nmol/mg
43
307
phosph., 1.71 mM
1.1 mg Mit
Rot., 5 µM
oli., 2 µg
CAT, 13 nmol/mg
CaCl2, 0.57 mM
P.A., 35 nmol/mg
25
252
CaCl2, 0.57 mM
P.A., 35 nmol/mg
arsenate 1.71 mM
1.1 mg Mit.
Rot. 5 µM
oli. 2 µg
CAT, 13 nmol/mg
ADP, 114 µM
CaCl2, 0.57 mM
P.A., 35 nmol/mg
33
267
DTE, 25 mM
41
ADP, 286 µM
44
DNP, 57 µM
44
134
DNP, 114 µM
44
120
DNP, 114 µM
31
86
DNP, 57 µM
42
98
DNP, 57 µM
41
87
100 ng atom O
1 min


ATPase (μ mol min⁻¹ mg protein⁻¹) (o—o)
0,4
0,2
15
10
5
Δ pH decay t1/2 (sec) (●—●)
2 min incub.
4 min incub.△
50
100
150
PA (μ M)

Legends

Fig. 1 Effect of picrylacetate on the coupled and arsenate stimulated succinate oxidation as compared to CAT and oli. Conditions: 10 mM succinate as substrate, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 mM KCl, 0.22 M mannitol, 70 mM sucrose, 2 mM hepes, pH 7.4; 30°C. The numbers on the lines are the oxygen uptake rates as nanogram atoms of oxygen per min per mg of protein.

Fig. 2 Effect of picrylacetate on succinate driven Ca^{2+}accumulation catalyzed by arsenate as compared with CAT and oli. Conditions as in Fig. 1, but without phosphate.

Fig. 3 Succinate driven Ca^{2+}accumulation catalyzed by phosphate or arsenate and effect of picrylacetate on the coupled succinate oxidation. Conditions as in Fig. 1, phosphate and arsenate as indicated.

Fig. 4 Effect of picrylacetate on succinate driven Ca^{2+}accumulation catalyzed by phosphate or arsenate. Conditions as in Fig. 3.

Fig. 5 Effect of picrylacetate on ATPase and ΔpH decay of coupled rat liver mitochondria. Conditions: ATPase activity spectrophotometrically at 30°C with 0.27 mg protein. O_2-pulse experiment in 0.22 M mannitol, 70 mM sucrose, 2 mM hepes, 10 mM succinate, 10 µg rotenone, 8 mg protein; pH 7.4, 30°C, N_2; O_2-pulse with O_2-saturated 0.25 M sucrose.