Communication

Direct Interaction of Mepacrine with Erythrocyte and Platelet Membrane Phospholipid*

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Mepacrine has been used as an inhibitor of the activation of endogenous phospholipases in many systems. These endogenous phospholipases are important in the modification of the lipid environment of membrane proteins and in the release of locally active oxygenated arachidonic acid metabolites. In both human platelets and erythrocytes, mepacrine blocks the release of fatty acid from phospholipid by endogenous phospholipases. However, mepacrine also interacts directly with membrane phospholipids, primarily phosphatidylethanolamine, to form less polar derivatives. This interaction occurs rapidly and is maximal at concentrations of mepacrine greater than 0.2 μM. Such drug-phospholipid interaction may perturb membrane architecture and function and be responsible for the inhibitory effects of mepacrine on cellular responses observed in many systems. Since the alteration in membrane phospholipid composition occurs under the same conditions as phospholipase inhibition, it is not possible to be certain that the inhibition of cellular responses by mepacrine is due to inhibition of phospholipases rather than to direct perturbation of the membrane. It is also possible that inhibition of phospholipase action by mepacrine is in part a consequence of the change in phospholipid composition. These results indicate that caution should be exercised in the interpretation of results obtained using mepacrine and that the usefulness of this compound for the investigation of the biological importance of phospholipase activation is limited.

Phospholipase activation is a critical step in the initiation of a variety of cellular responses (1-22). Phospholipases modify membrane phospholipid composition in situ, by (a) generating hydrolysis products, i.e., lysophospholipid and diacylglycerol, which have different physical properties than intact phospholipids (23, 24) and (b) permitting modification of membrane phospholipid fatty acid composition via an endogenous deacylation-reacylation pathway (25, 26). Both of these metabolic events can affect the physical properties of membrane lipids and influence the local environment of membrane proteins. Such changes in lipid microenvironment may be important in the transduction of receptor-mediated changes in cellular function or in the control of membrane functions such as transport, phagocytosis, or secretion (23-26). In addition to their potential role in modification of membrane lipid composition, phospholipases also govern the availability of arachidonic acid which is released from membrane phospholipids and utilized for the biosynthesis of highly active oxygenated derivatives by cyclooxygenase and lipoxygenase (1-4, 12-22).

Many recent studies have utilized putative phospholipase inhibitors in an attempt to demonstrate the importance of phospholipase activation in the initiation of cellular responses (1-14). One agent which has been extensively employed in recent studies is mepacrine (quinacrine, N(6-chloro-2-methoxy-9-acridinyl)-N(7),N(10)-diethyl-1,4-pentanediamine). Mepacrine has been shown to block release of fatty acids from phospholipid, a measure of phospholipase A2 activity, in several different systems including platelets (3, 11, 12) spleen (2), toad bladder (5), leukocytes (6), astrocytoma (7), colonic mucosa (8), and pituitary (9). While it is recognized that mepacrine may have other actions, it has been assumed that its ability to block cellular responses is due to inhibition of the phospholipase thought to be involved in these processes. During the course of studies of erythrocyte and platelet membrane phospholipid metabolism, we have observed that, in addition to apparently inhibiting endogenous phospholipase activity, mepacrine also interacts directly with membrane phospholipids to form less polar derivatives. It is possible that these interactions could directly perturb membrane architecture and function. Additionally, when the presence of these phospholipid derivatives is not taken into consideration, correct determination of membrane phospholipid composition is not possible. Thus, since mepacrine has direct effects on membrane phospholipid, it would appear to have limited usefulness as a selective phospholipase A2 inhibitor in the investigation of the role of endogenous phospholipase activation in the control of cellular processes.

EXPERIMENTAL PROCEDURES

Endogenous phospholipase activity in human erythrocytes was determined by measuring release of [9,10-3H]oleic acid (Amersham-Searle) from prelabeled erythrocyte membranes prepared by hypotonic hemolysis (27). Lipid extraction and phosphorus analysis were carried out as previously described (26, 27). Neutral lipids were separated on a thin layer of silica G (Supelco) in hexane-ether-acetic acid, 70:30:1 (v/v). Phospholipid separation was performed on a thin layer of silica H (Sigma) prepared with 1 mM Na2CO3, and developed sequentially in ether-acetic acid, 98:2 (v/v) to separate neutral and phospholipids followed after drying for 10 min with chloroform-methanol-acetic acid-water, 50:20:5:2 (v/v). Phospholipids were visualized by exposure of thin layer plates to iodine. This system separates sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine. Phosphatidylinerine and phosphatidyllysinomaltolate migrate together but are separated from the other phospholipids.

Human platelets were isolated (28) and prelabeled with [1-14C]arachidonic acid (19). Parallel samples were incubated at 37 °C in a buffer composed of 140 mM NaCl, 15 mM Tris, 5.5 mM glucose, pH 7.4, without agitation for 15 min in the presence and absence of mepacrine (Sigma) and then treated with bovine thrombin (5 units/ml) (Parke-Davis) for 10 min. Lipid extraction and phosphorus analysis were carried out using the same procedures employed for erythrocyte membranes (27). Oxygenated derivatives of [1-14C]arachidonic acid were extracted from the incubation medium with three equal volumes of ethyl acetate following acidification to pH 3 with HCl. Separation of cyclooxygenase and lipoxygenase products derived...
from arachidonic acid was carried out by silica gel thin layer chromatography in the organic phase of ethyl acetate-acetic acid: iso-octane-water, (90:20:50:100) (v/v) (29). This system separates thromboxane B2, and 12-1-hyroxy-5,8,10,14-eicosatetraenoic acid from arachidonic acid and phospholipids.

**RESULTS**

**Effects of Mepacrine upon Human Erythrocyte Membrane Phospholipids**—Incubation of erythrocyte membranes with increasing concentrations of mepacrine resulted in progressive inhibition of release of [9,10-3H]oleic acid from phospholipid, (Fig. 1). Since [9,10-3H]oleic acid is present only in phospholipid after prelabeling, release of free [9,10-3H]oleic acid from isolated erythrocyte membranes is a measure of the endogenous phospholipase previously detected (26, 27). When the composition of erythrocyte membranes is examined by measuring lipid phosphorus following exposure to mepacrine, several fluorescent phosphorus-containing components, not present in unexposed membranes, are detected after thin layer chromatographic separation of membrane phospholipids (Figs. 2 and 3). The major component of PL-M migrates with $R_f = 0.9$ in the second step of the phospholipid thin layer chromatographic system while mepacrine itself, which is fluorescent but does not contain phosphorus, has $R_f = 0.4$. PL-M has a greater $R_f$ than most native erythrocyte phospholipids indicating that it is less polar. These fluorescent components co-migrate with phosphatidic acid and other minor phospholipids in the acidic thin layer chromatographic system employed in this study. This accounts for the presence of lipid phosphorus with $R_f$ similar to PL-M in membranes not exposed to mepacrine (i.e. at $t = 0$). PL-M can be distinguished from PA because it also has a greater $R_f$ than native erythrocyte phospholipids in a basic thin layer chromatographic system (chloroform:methanol:ammonium hydroxide, 65:35:5 (v/v)) where PA has $R_f$ less than 0.1. While most of the PL-M is formed during the first 20 min of incubation, additional PL-M accumulates during incubation to 60 min (Fig. 2). No change in the content of the less polar native phospholipids co-migrating with PL-M occurs during this period. The dependence of the appearance of PL-M on the time of incubation suggests that formation of PL-M does not simply reflect interaction between mepacrine and native phospholipids occurring during extraction. The appearance of these phospholipid derivatives thus reflects direct interaction between membrane phospholipid and mepacrine. Formation of PL-M is also dependent upon the concentration of mepacrine in the incubation medium (Figs. 2 and 3) with maximal interaction occurring at concentrations of mepacrine above 0.2 mM. Inhibition of endogenous phospholipase by mepacrine is not maximal at this concentration (Fig. 1). These concentrations of mepacrine are comparable to those employed in other systems where inhibition of both phospholipases and cellular responses are observed (1-10, 12-14). The appearance of PL-M is accompanied by a reduction in the amounts of PE and PS/PI (Fig. 3). No significant change in the amount of PC/S or net increase in total phospholipid is detected. PE undergoes the most striking reduction in amount. A smaller decrease in the amount of PS/PI is also detectable. The sum of these reductions account for all of the PL-M formed. Thus, PL-M appears to be derived from interactions between mepacrine and native phospholipids and does not reflect formation of phospholipid de novo.

Effects of Thrombin and Mepacrine on Human Platelets—Treatment of [1-14C]arachidonic acid labeled human platelets with thrombin results in release of [1-14C]arachidonic acid from PS/PI and PC and formation of [14C]12-HETE and [14C]TxB2 by the combined actions of endogenous phospholipase and either lipooxygenase or cyclooxygenase (Fig. 4). This release of [1-14C]arachidonic acid is accompanied by a small decrease in the mass of PC but no detectable decrease in the mass of PS/PI. Mepacrine (0.5 mM) prevents the thrombin-induced loss of [1-14C]arachidonic acid from PC and PI and the formation of [1-14C]12-HETE and [1-14C]TxB2 (Fig. 4) (12-14). These results are consistent with mepacrine-induced inhibition of endogenous erythrocyte membrane phospholipase. Human erythrocyte membranes prelabeled with [9,10-3H]oleic acid and isolated by hypotonic hemolysis as described were incubated at 37 °C in buffer containing 35 mM KC1, 5 mM NaCl, 3 mM MgSO4, and 10 mM Tris, pH 7.4, and varying concentrations of mepacrine. [9,10-3H]oleic acid released as free fatty acid was measured following lipid extraction and thin layer chromatography of neutral lipids as described under "Experimental Procedures."
inhibition of an endogenous phospholipase although inhibition of oxygenases is not excluded (3). Treatment of platelets with mepacrine (0.5 mM) in the presence or absence of thrombin results in a decrease in [1'-14C]arachidonate in PE and in the mass of PE measured as lipid phosphorus. This is accompanied by an increase in [1-14C]arachidonic acid and lipid phosphorus associated with less polar fluorescent phospholipids which show thin layer chromatographic behavior identical to that of PL-M detected in erythrocytes. In the platelet, as in the erythrocyte, most of the PL-M is accounted for by a decrease in PE (Fig. 4). Thus, in both platelets and erythrocytes, exposure to mepacrine prevents loss of fatty acid from membrane phospholipids and results in formation of phospholipids derived from interactions of mepacrine with membrane PE.

**DISCUSSION**

The results of this study indicate that mepacrine, an antimalarial drug which has been utilized as a phospholipase A2 inhibitor in many systems (1-14), interacts directly with both erythrocyte and platelet membrane phospholipids. The exact nature of this interaction which involves primarily PE and results in formation of several less polar derivatives is unknown. The existence of such interactions was initially suggested in early studies of mepacrine metabolism in which phosphorus containing lipid soluble compounds containing mepacrine were isolated from mouse liver following oral administration (30). These compounds which were stable to dilute acid were thought to represent mepacrine phospholipid salts although their precise composition was not clearly defined. Under the same conditions that result in formation of mepacrine-phospholipid derivatives in both platelets and erythrocytes, mepacrine inhibits endogenous phospholipase activity (Figs. 1 and 4). Although mepacrine has been shown previously to prevent fatty acid release from membrane phospholipids in several systems (2, 3, 6-9), the formation of phospholipid derivatives similar to those observed in this study has not been described. Detection of such derivatives would require measurements of phospholipid distribution. Such measurements have not been carried out in studies using mepacrine as a putative phospholipase inhibitor.

The mechanism of action of mepacrine is not clear. It was initially developed as an antimalarial and antibiotic. The basis for these activities has not been fully elucidated although effects on a variety of cellular functions have been described (31, 32). The use of mepacrine as a phospholipase inhibitor was initially based on studies of hormone-mediated lipolysis by adipocytes (33) where relatively high concentrations of mepacrine (1-6 mM) were observed to inhibit both hormone-stimulated and basal rates of fatty acid release. The substrate for lipolysis in this study was not identified. In all likelihood, it was neutral lipid rather than phospholipid. Nonetheless, this study is subsequently cited as the basis for use of mepacrine as a phospholipase inhibitor, although the authors of the original study (33) did not make this claim. Mepacrine has since been shown to block release of fatty acids from phospholipids in a variety of systems where it also inhibits a variety of cellular responses (2, 3, 5-9, 11, 12). However, the results of the present study suggest that alterations in membrane phospholipid architecture by mepacrine-phospholipid interactions rather than direct interaction of mepacrine with phospholipases could also be responsible for the inhibition of cellular responses by mepacrine. It is also possible that these mepacrine-phospholipid interactions are involved in the inhibition of phospholipase activity although no direct effects of PL-M on phospholipase activity have been detected in this study. Comparison of Figs. 1 and 2 shows that the dependence of mepacrine effects on mepacrine concentration is different. No direct demonstration of inhibition of an isolated phospholipase by mepacrine has been described. A recent study compared mepacrine to a series of compounds with inhibitory activity in an in vitro phospholipase-lipoxygenase coupled assay (34). It is possible that the inhibition of phospholipase activity by mepacrine observed in this in vitro assay system resulted from direct interactions between mepacrine and the phospholipid substrate. Thus, the occurrence of direct drug-phospholipid interactions must be considered when mepacrine is used as a phospholipase inhibitor.

In several recent studies of platelet activation (12-14), mepacrine has been utilized as a selective phospholipase A2 inhibitor. Since the relative importance of phospholipase A2 and phospholipase C in the release of arachidonic acid from platelet phospholipids is currently a matter of considerable debate (18, 20-22), proper interpretation of these studies is critical. In one study, an experiment similar to that described in Fig. 4 of the present study was performed with horse platelets (12). In this experiment, thrombin resulted in the release of [1-14C]arachidonic acid from PC, PI, and PE and the incorporation of [1-14C]arachidonic acid into phosphatidic acid. The remainder of the [1-14C]arachidonic acid released was detected as oxygenated products. A net decrease in the mass of PI and an increase in the mass of PA occurred. Mepacrine prevented the thrombin-induced release of [1-14C]arachidonic acid from PC, PI, and PE and the appearance of oxygenated products while actually increasing the formation of PA detected by either [1-14C]arachidonate incorporation or lipid phosphorus analysis. These results were interpreted as supporting the hypothesis that the phospholipase C-mediated conversion of PI to PA is the initial effect of thrombin on platelet phospholipids rather than release of [1-14C]arachidonic acid from PC, PE, and PI by phospholipase A2. Subsequent studies by Billah et al. (13) and Lapetina et al. (14) have extended this hypothesis by examining metabolism of 32P-phospholipids without measuring lipid phosphorus in mepacrine treated platelets exposed to thrombin. However, the effects of mepacrine alone on the distribution of [1-14C]arachidonic acid or phospholipid phosphorus have not been described in any of their studies. Because their experiments were conducted under conditions similar to those employed in the present study, it is possible that direct interaction of
mepacrine with platelet phospholipids rather than selective inhibition of phospholipase A₂ is responsible for the observed effects of mepacrine on thrombin-treated horse platelets. The failure of mepacrine to prevent the increase in PA induced by thrombin could result from measurement of PL-M as PA since PL-M and PA have similar polarity in acidic thin layer chromatographic systems. In the present study, [1-¹⁴C]arachidonic acid incorporation into PA could be detected in thrombin-treated human platelets (Fig. 4). This [1-¹⁴C]arachidonyl PA comigrates with PL-M in the acidic thin layer chromatographic system employed in this study but can be distinguished from it by thin layer chromatography in a basic system. In platelets exposed to mepacrine in the presence or absence of thrombin, there is a significant increase in both [1-¹⁴C]arachidonic acid and lipid phosphorus which is associated with the formation of PL-M rather than PA. This amount of [1-¹⁴C]arachidonoyl PL-M formed is similar to the amount of [1-¹⁴C]arachidonoyl PA formed by thrombin-treated platelets and may account for the observation that mepacrine actually increased the apparent formation of PA by thrombin in the absence of thrombin, there is a significant increase in both [¹-¹⁴C]arachidonic acid and lipid phosphorus which is associated with the formation of PL-M rather than PA. This amount of [1-¹⁴C]arachidonoyl PL-M formed is similar to the amount of [1-¹⁴C]arachidonoyl PA formed by thrombin-treated platelets and may account for the observation that mepacrine actually increased the apparent formation of PA by thrombin in the study of Lapetina et al. (12). Thus, the increase in PA detected in thrombin-treated horse platelets exposed to mepacrine by these investigators could actually reflect PL-M formed by interactions similar to those observed with human platelets in this study rather than lack of inhibition of a thrombin-induced activation of the phospholipase C-diglyceride kinase cycle.

The results presented in this report demonstrate direct interactions between mepacrine and membrane phospholipids in both erythrocytes and platelets. Consequently, this effect of mepacrine must be considered whenever this compound is employed in phospholipid-containing systems. The utility of drug-phospholipid interaction, are performed.

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