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Triphenylmethylphosphonium Cation Distribution as a Measure of Hormone-Induced Alterations in White Adipocyte Membrane Potential

Mary Lou Vallano and Martin Sonenberg

Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York 10021

Summary. Triphenylmethylphosphonium ($TPMP^+$) partitions into the mitochondrial and cytosolic compartments in the rat white adipocyte in a potential-dependent fashion. The relationship between [^{3}H]$TPMP^+$ distribution, intracellular cAMP generation and lipolysis in response to hormones and cAMP-mimetic compounds was examined. Half-maximal [^{3}H]$TPMP^+$ efflux and glycerol release were produced by 15 and 9 nM adrenocorticotropin, 170 and 110 nM l-epinephrine, 70 and 27 μM isobutylmethylxanthine and 800 and 750 μM dibutyryl cAMP, respectively. Hormone-stimulated cAMP generation was also correlated with [^{3}H]$TPMP^+$ efflux and lipolysis in terms of concentration dependency. In kinetic experiments, glycerol release and [^{3}H]$TPMP^+$ efflux in response to adrenocorticotropin or cholera toxin proceeded over a similar time course, whereas an earlier rise in cAMP generation was detected.

The depolarizing effect of lipolytic compounds was localized to the mitochondrial compartment. When cells were incubated in elevated-$[K^+]_o$ buffer, the stimulatory effect of dibutyryl cAMP on [^{3}H]$TPMP^+$ efflux and lipolysis persisted, suggesting that maintenance of the plasma membrane potential is not critical for demonstration of these responses.

When the extracellular concentration of serum albumin, which provides binding sites for free fatty acids, was increased from 1 to 3%, an increase in glycerol release and a decrease in [^{3}H]$TPMP^+$ efflux was observed. We suggest that intracellular free fatty acid accumulation in response to lipolytic agents causes dissipation of the mitochondrial membrane potential and efflux of [^{3}H]$TPMP^+$ from the organelle and cell.

Key words triphenylmethylphosphonium · membrane potential · adipocyte · mitochondria · lipolysis

Introduction

The plasma membrane of the rat white adipocyte contains receptors for a variety of lipolytic hormones that activate adenylate cyclase [6] including β-adrenergic agonists [53] and ACTH[1] [32]. Interaction between hormone and receptor rapidly leads to the generation of intracellular cAMP [7], resulting in the activation of a cAMP-sensitive protein kinase [14]. Protein kinase catalyzes transfer of the terminal phosphate group from ATP to triglyceride lipase, the rate-limiting enzyme that cleaves FFA from glycerol [13, 15]. Glycerol and FFA are subsequently released into the medium where FFA are noncovalently bound to serum albumin. Accumulation of FFA causes a reduction in the rate of lipolysis by adipose tissue [50] and isolated adipocytes [46]. When the molar ratios of medium FFA/albumin exceed 3:1, adenylate cyclase and triglyceride lipase are inhibited [20, 34]. Therefore, it was suggested that FFA serve as negative feedback regulators of lipolysis, although the mechanism of regulation is uncertain [for review, *see* 21].

Free fatty acids may indirectly inhibit lipolysis by altering mitochondrial function. The ADP-phosphorylating and calcium-accumulating capacities of the mitochondria are strictly dependent upon energization of the inner mitochondrial membrane [47, 48]. A reduction in cytosolic ATP levels and/or an increase in free cytosolic calcium levels resulting from deenergization of the membrane by FFA could interfere with cyclase and lipase activities [31, 40]. In isolated mitochondria, FFA uncouple oxidative phosphorylation, dissipate the mitochondrial protonmotive force and increase mitochondrial calcium efflux [4, 43]. Recently, ψ_m has been measured in intact cells with potential-sensitive probes [18, 19, 25], thus providing a possible means of examining the effects of FFA on ψ_m in the isolated adipocyte.

In a preliminary report, we described the accumulation of [^{3}H]$TPMP^+$ by the intact rat white adipocyte as an index of plasma membrane potential assuming probe partitioning into a single compartment [9]. In addition, the effects of catecholamines

[1] *Abbreviations:* triphenylmethylphosphonium, $TPMP^+$; plasma membrane potential, ψ_p; mitochondrial membrane potential, ψ_m; adipocyte membrane potential, ψ_t; adrenocorticotropin, ACTH; isobutymethylxanthine, IBMX; adenosine 3′,5′-cyclic monophosphate, cAMP; guanosine 3′,5′-cyclic monophosphate, cGMP; adenosine diphosphate, ADP; adenosine triphosphate, ATP; dibutyryl cAMP, dibutyryl cGMP, Bt_2cAMP, Bt_2cGMP; extracellular concentration of x, $[x]_o$; intracellular concentration of x, $[x]_i$; bovine serum albumin, BSA; free fatty acids, FFA; *m*-chloro-carbonylcyanide-phenylhydrazone, CCCP; nicotinamide adenine dinucleotide, NADH.

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and insulin on [^{3}H]TPMP$^+$ distribution in the cell were described [10]. Attempts to analyze the distribution of [^{3}H]TPMP$^+$ into intact hepatocytes [25], neuroblastoma [19] and adipocytes [18] have indicated that a substantial amount of the probe partitions into the mitochondria according to ψ_m, whereas a much smaller fraction accumulates in the cytosolic compartment. In the present report, we provide additional evidence for the subcellular distribution of [^{3}H]TPMP$^+$ into both the mitochondrial and cytoplasmic compartments in the intact adipocyte. The relationships between intracellular cAMP generation, [^{3}H]TPMP$^+$ efflux and glycerol release in response to lipolytic hormones and cAMP-mimetic compounds were examined. The possibility that intracellular free fatty acid accumulation in response to lipolytic agents produces [^{3}H]TPMP$^+$ efflux from the mitochondria and subsequently from the adipocyte was also investigated.

Materials and Methods

Adipocyte Preparation

Adipocytes were prepared by digesting minced epididymal fat tissue in crude collagenase as previously described [9] according to a modification of the method of Rodbell [45]. After washing, the cells were resuspended to a final concentration of 20% (wt/vol) in Tris HCl buffer [124.0 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM KH_2PO_4, 24.6 mM Tris HCl, 1.0 mM glucose, 1% (wt/vol) BSA (Fraction V), pH 7.4]. Changes in buffer composition that were used in ion substitution and BSA experiments are described in the text.

[^{3}H]TPMP$^+$ Uptake Experiments

The uptake of [^{3}H]TPMP$^+$ by isolated adipocytes was initiated upon addition of 1 volume of the cell suspension to 9 volumes of Tris HCl buffer (prewarmed to 37 °C) containing 1 μM [^{3}H]TPMP$^+$ (3.6 μCi) and the test compounds or vehicle. Unless indicated otherwise, cells were incubated for 60 min at 37 °C prior to termination of the uptake reaction. [^{3}H]TPMP$^+$ was previously shown to reach equilibrium after a 30-min incubation period [9]. The cells were separated from the incubation medium and cell-associated radioactivity was determined as previously described [9].

Glycerol Release Experiments

Glycerol release studies were performed in parallel with [^{3}H]TPMP$^+$ uptake experiments. The incubation conditions were identical except that [^{3}H]TPMP$^+$ was not present in the medium[2]. Glycerol levels were determined fluorometrically [11]. The assay is based upon the stoichiometric generation of NADH (the measured product) from glycerol in two successive enzymatic steps: conversion of glycerol to glycerol-3-phosphate in the presence of ATP and glycerokinase and the subsequent conversion of glycerol-3-phosphate to dihydroxyacetone phosphate + NADH in the presence of NAD$^+$ and glycerophosphate dehydrogenase. The assay sensitivity and reproducibility were improved by including 5 mM dithiothreitol in the assay medium so that a lower limit of 0.5 nmol glycerol was detectable.

Cell number/sample was quantitated by counting a dilution of the original 20% cell suspension under a light microscope. On the average, 250,000 cells/ml buffer were present in each sample tube.

Measurement of Intracellular cAMP Generation

Dynamic changes in adipocyte cAMP levels in response to lipolytic hormones were measured by a modification of a previously described method [30]. The adipocyte ATP pool was pulse-labeled by incubating the cells with the permeable precursor, [^{3}H]adenine. One volume of packed cells was incubated with 1 volume of Tris HCl buffer containing 3% BSA and [^{3}H]adenine (100 μCi) at 37 °C for 30 min. The preloaded adipocytes were washed three times in buffer and resuspended to a 20% (wt/vol) cell suspension in buffer containing 1% BSA prior to use. The incubation conditions for the determination of cAMP and glycerol release were identical except for the length of incubation. The reaction was terminated after 5 min by adding 10 volumes of sample to 1 volume of 5 N perchloric acid. [^{3}H]cAMP was separated from other radiolabeled compounds in the sample by two sequential column chromatographic steps [12]. The samples were concentrated to dryness in a vortex evaporator (Buchler), dissolved in 1 ml of H_2O, and the radioactivity was determined by liquid scintillation spectrometry at 34% efficiency in 10 ml of Formula 963 scintillation cocktail. Percent recovery of [^{3}H]cAMP was measured in a parallel set of samples and was ≃70%.

Materials

Male Sprague-Dawley rats (150–200 g) were purchased from Charles River Laboratories. [^{3}H]TPMP$^+$ (sp act: 3.59 Ci/mmol), [^{3}H]adenine (sp act: 16.6 Ci/mmol) and Formula 963 scintillation cocktail were purchased from New England Nuclear Corp. Crude bacterial collagenase (Type 1) was obtained from Worthington Biochemical Corp. Unlabeled TPMP$^+$, dinonyl phthalate and l-epinephrine were purchased from K & K Laboratories. Bovine serum albumin (Fraction V), oligomycin, dinitrophenol, ouabain, antimycin A, valinomycin, porcine adrenocorticotropin (Grade II), glycerokinase (EC 2.7.1.30), α-glycerophosphate dehydrogenase (EC 1.1.1.8), dibutyryl cAMP, dibutyryl cGMP, isobutylmethylxanthine and dithiothreitol were obtained from Sigma Chemical Co. *m*-chlorocarbonylcyanide-phenylhydrazone was obtained from U.S. Biochemical Corp. and was further purified by recrystallization from absolute ethanol. Cholera enterotoxin (lot #0972) was generously supplied by Dr. R. Finkelstein from Southwestern Medical School in Dallas, Texas. The cholera toxin employed was prepared under contract for the National Institute of Allergic and Infectious Diseases, National Institutes of Health, as previously described [23]. All other compounds were reagent grade, obtained from commercial sources.

Results and Discussion

I. Subcellular Distribution of [^{3}H]TPMP$^+$

The relative distribution of [^{3}H]TPMP$^+$ into the cytoplasmic and mitochondrial compartments was

[2] In control experiments, 1 μM TPMP$^+$ did not interfere with ACTH-or epinephrine-stimulated glycerol release, whereas a 10-fold greater concentration of TPMP$^+$ inhibited hormone-stimulated glycerol release to some extent (≃20–30%).

Table 1. Effects of inhibitors and ionophores on the steady-state distribution of $TPMP^+$ into adipocytes

Treatment		% Cell-associated $TPMP^+$
Control		100.0
Antimycin A	(5 μM)	17.1±1.0
KCN	(2 mM)	18.7±0.8
Dinitrophenol	(100 μM)	15.6±0.2
CCCP	(5 μM)	9.8±0.5
Valinomycin	(2 μM)	10.6±0.5
Oligomycin	(5 μM)	73.5±4.3
Ouabain	(1 mM)	93.7±0.3

Cells were incubated with test compounds or vehicle (all compounds were dissolved in ethanol, except KCN) for 60 min at 37 °C in Tris HCl buffer. The maximal inhibitory concentration observed with each compound is reported. The data are expressed as: % cell-associated $TPMP^+$ compared to 100% control uptake (100% control uptake = the total amount of cell-associated $TPMP^+$). Reported values represent the mean ±SEM for three separate determinations. Less than 2% of the $[TPMP^+]_o$ was cell-associated [for details, *see* Appendix].

evaluated in the presence of a variety of mitochondrial inhibitors that eliminate ψ_m. The maximal inhibitory effects of mitochondrial uncoupling agents, ionophores and ouabain on the steady-state distribution of $[^3H]TPMP^+$ into adipocytes are shown in Table 1. Antimycin A, which interrupts electron flow from cytochrome *b*, reduced cell-associated $[^3H]TPMP^+$ by 83%. KCN, which inhibits reoxidation of cytochrome oxidase, produced an 81% loss of the probe. The prototypic uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, diminished $[^3H]TPMP^+$ uptake into adipocytes by 84%. The largest reductions in cell-associated probe, 90 and 89%, were produced by CCCP and valinomycin, respectively. All of these agents dissipate the mitochondrial electrochemical potential and reduce cytosolic ATP levels [29, 42]. A secondary inhibition of the plasma membrane Na^+-K^+ ATPase could result from the reduction in cellular ATP levels upon addition of mitochondrial inhibitors and contribute to the observed loss of $[^3H]TPMP^+$. However, two experimental observations suggest otherwise: (i) Oligomycin prevents ADP-phosphorylation and thereby reduces cytosolic ATP levels through direct inhibition of the ATP synthetase [33], but the proton gradient that is generated across the inner mitochondrial membrane by oxidation is affected minimally. As expected, oligomycin had a marginal effect on adipocyte $[^3H]TPMP^+$ distribution compared to the agents that abolish ψ_m, despite the fact that cytosolic ATP levels are diminished by all of the compounds. Moreover, the slight reduction in adipocyte potential (ψ_t) that was observed in response to oligomycin persisted when the cells were incubated in elevated-$[K^+]_o$ buffer to eliminate ψ_p, indicating that the probe was lost from the mitochondrial compartment *(data not shown)*. (ii) We have previously reported that ψ_p in the adipocyte is generated by a K^+-diffusion potential and not by the electrogenic activity of the Na^+-K^+ ATPase [9]. Incubation of adipocytes with 1 mM ouabain for 60 min diminished cell-associated $[^3H]TPMP^+$ by only 6%. Since direct inhibition of the plasma membrane Na^+-K^+ ATPase by ouabain did not reduce ψ_p to a large extent, it may be argued that the reduction in cytosolic ATP levels resulting from dissipation of ψ_m should not diminish ψ_p to a large extent.

In a multicompartment system, the intracellular accumulation of a lipophilic cation is related to the volume fraction of the compartment and the electrical potential difference existing across the membrane that delineates the compartment. Since most cells maintain significant potential differences across the plasma and inner mitochondrial membranes, $[^3H]TPMP^+$ accumulates into both the cytosolic and mitochondrial compartments in a manner that reflects ψ_p and ψ_m [2, 47]. Lysosomes may develop an electrochemical potential due to a low intralysosomal pH, but the small volume fraction and the permeability characteristics of this organelle at physiological temperatures would minimize its contribution to the total amount of accumulated probe [25, 44]. Nuclei would not be expected to accumulate a lipophilic cation since an electrical potential was not detected across the nuclear membrane [39].

In the present report, the adipocyte will be treated as a two-compartment system with regard to potential-sensitive probe accumulation. Precise determinations of ψ_p and ψ_m by the method of the $TPMP^+$ distribution are complicated due to the difficulty associated with measuring compartmental volumes in a cell with a limited H_2O space [18, 27, 38]. Where possible, determination of ψ_p and ψ_m with potential-sensitive probes should be used in conjunction with independent methods of monitoring ψ's such as electrophysiological recording. Nevertheless, meaningful information concerning the role of membrane potentials in adipocyte responsiveness to hormones can be derived from potential-sensitive probe experiments.

Since the electrochemical gradient of hydrogen ions across the mitochondrial inner membrane generates ψ_m [36, 37], we felt that a protonophore was the most specific agent with which to eliminate the mitochondrial component of ψ_t. Therefore, ψ_m has been defined as that portion of the cell-associated probe which is sensitive to 5 μM CCCP ($\simeq$90%), assuming no direct or indirect effect of CCCP on ψ_p [35]. The $[^3H]TPMP^+$ that remains associated with

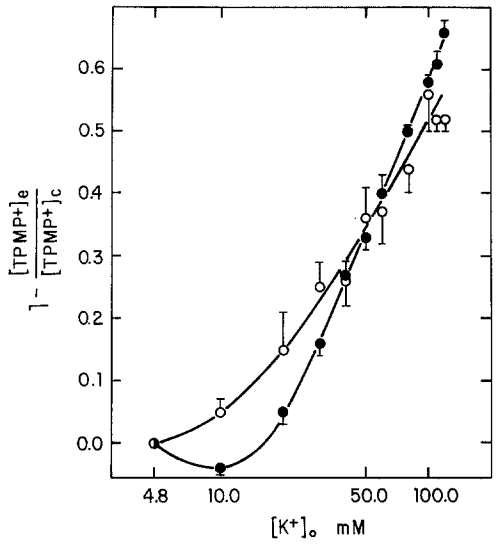

Fig. 1. Effect of $[K^+]_o$ on $[^3H]TPMP^+$ distribution into adipocytes. Cells were incubated for 60 min at 37 °C in Tris HCl buffer containing the indicated $[K^+]_o$ (replacing $[Na^+]_o$) in the absence (●) or presence (○) of 5 μM CCCP. The fraction of probe that was lost from the cells is expressed as:

$$1-\frac{[TPMP^+]_e}{[TPMP^+]_c}=1-\frac{[TPMP^+]_{experimental}}{[TPMP^+]_{control}}$$

($[TPMP^+]_c$ = the total amount of cell-associated $TPMP^+$ ± CCCP). In the absence of a perturbant, $[TPMP^+]_e=[TPMP^+]_c$ so that $1-\frac{[TPMP^+]_e}{[TPMP^+]_c}=0$. All points represent the mean ± SEM of three separate experiments

the cell in the presence of 5 μM CCCP and responds to changes in $[K^+]_o$ and $[Na^+]_o$ has been defined as ψ_p. When $\psi_t=0$, $[TPMP^+]_o=[TPMP^+]_i$ minus the amount of probe that is nonspecifically bound to cellular components, so that an absolute amount of the probe will remain associated with the cell in the absence of an electrical potential [*refer to* Appendix].

II. Effects of Extracellular Ion Substitutions on $[^3H]TPMP^+$ Distribution

In a previous report, we concluded that the plasma membrane of the rat white adipocyte is predominantly permeable to K^+ and Na^+, and that the resting potential is due to the cation-diffusion potential [9]. The data shown in Fig. 1 and Table 2 corroborate that report. Gradual replacement of $[Na^+]_o$ with $[K^+]_o$ produced a $[K^+]$-concentration-dependent efflux of $[^3H]TPMP^+$, i.e., *adipocyte depolarization* in both the presence and absence of CCCP. In the absence of CCCP, K^+-induced efflux

Table 2. Effects of extracellular ion substitutions on the steady-state distribution of $TPMP^+$ into adipocytes

	% Cell-associated $TPMP^+$	
Buffer	+Vehicle	+5 μM CCCP
Normal	100.0	100.0
Na^+-free	127.4±4.0	203.9±9.0
Cl^--free	95.0±5.1	89.9±3.0
Mg^{2+}-free	108.2±1.3	89.9±3.2
Ca^{2+}-free	91.5±1.5	91.0±6.0

Cells were incubated for 60 min at 37 °C in normal or modified Tris buffers (±5 μM CCCP). Osmolarity (314.8 mOsm/liter) was maintained in all substitution experiments. In Na^+-free buffer, $[NaCl]_o$ was replaced with sucrose. In Cl^--free buffer, $[NaCl]_o$ and $[KCl]_o$ were replaced by sodium and potassium methylsulfate; $[CaCl_2]_o$ was replaced by $[Ca(OH)_2]_o$; and Tris HCl was replaced with Tris SO_4. $[CaCl_2]_o$ or $[MgSO_4]_o$ were replaced with $[NaCl]_o$. The data are expressed as: % cell-associated $TPMP^+$ compared to 100% control uptake (100% control uptake = the total amount of cell-associated $TPMP^+$ ± CCCP). Reported values represent the mean ± SEM for three separate determinations.

of the probe reflects depolarization of the plasma membrane since the inner mitochondrial membrane is normally impermeable to K^+ [26]. However, the mitochondrial $[^3H]TPMP^+$ will reequilibrate in response to the imposed reduction in cytoplasmic $[^3H]TPMP^+$ and at maximally depolarizing $[K^+]_o$, a specific amount of $[^3H]TPMP^+$ will remain associated with the mitochondria due to ψ_m. Addition of valinomycin to the incubation medium released the remainder of the potential-sensitive probe, presumably by increasing the permeability of the inner mitochondrial membrane to K^+ [26]. When ψ_m is eliminated by incubating the cells with CCCP, the probe that remains associated with the adipocyte responds to an elevation in $[K^+]_o$ in a manner indicative of plasma membrane depolarization. As expected, replacement of $[Na^+]_o$ with equiosmolar sucrose produced an increase in cell-associated $[^3H]TPMP^+$, i.e., *hyperpolarization* in the presence and absence of CCCP (Table 2). Replacement of $[Mg^{2+}]_o$, $[Ca^{2+}]_o$ or $[Cl^-]_o$, did not significantly affect $[^3H]TPMP^+$ distribution. Thus, the adipocyte plasma membrane is primarily permeable to K^+ and Na^+. A detailed report of these and other ion substitution experiments will be published separately[3]. If some assumptions are made with regard to cytoplasmic and mitochondrial volumes in the adipocyte, then ψ_p and ψ_m can be approximated [*refer to* Appendix]. The ψ_m ranged from −167 to −178 mV, a

[3] M.L. Vallano, M.Y. Lee, B. Osotimehin and M. Sonenberg, *manuscript in preparation.*

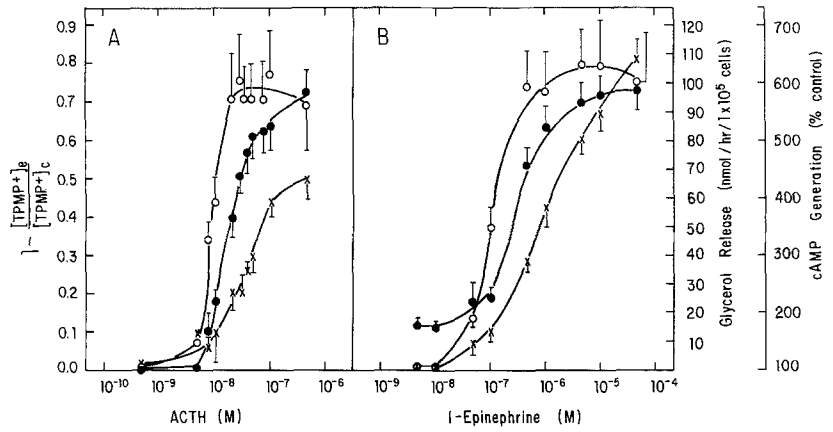

Fig. 2. Effects of ACTH and l-epinephrine on $[^3H]TPMP^+$ distribution, intracellular cAMP generation and lipolysis. Cells were incubated with hormone or vehicle for 60 min ($[^3H]TPMP^+$ and glycerol experiments) or 5 min (cAMP experiments) at 37 °C in Tris HCl buffer. $[^3H]TPMP^+$ distribution (●): glycerol release above basal (○; basal glycerol release was 5–15 nmol/hr/1 × 10^5 cells): % intracellular cAMP generation (×). All points represent the mean ± SEM of at least three separate determinations

value that compares favorably with other estimates of ψ_m in the adipocyte [−152 mV, 18] and the hepatocyte [−161 mV, 25]. Adipocyte ψ_p ranged from −30 to −40 mV. A ψ_p value of −29 mV has been reported based on the Cl^- distribution ratio [41] and −75 mV as determined by the $^{86}Rb^+$ diffusion potential [18] in isolated adipocytes.

III. Effects of Hormones and cAMP-mimetic Compounds on $[^3H]TPMP^+$ Distribution

The effects of increasing concentrations of ACTH and epinephrine on $[^3H]TPMP^+$ distribution, cAMP generation and glycerol release are shown in Fig. 2. There is a direct correlation between the lipolytic potencies of ACTH and epinephrine and their abilities to produce $[^3H]TPMP^+$ efflux from the cell. Half-maximal glycerol release was observed in response to 9 nM ACTH and 110 nM epinephrine and half-maximal $[^3H]TPMP^+$ efflux was elicited by 15 nM ACTH and 170 nM epinephrine. Good agreement between hormone-stimulated intracellular cAMP generation, $[^3H]TPMP^+$ efflux and glycerol release was observed for both lipolytic hormones.

To determine whether hormone-stimulated $[^3H]TPMP^+$ efflux may be mediated by an increase in intracellular cAMP, the effects of two cAMP-mimetic agents on $[^3H]TPMP^+$ efflux and glycerol release from the adipocyte were examined as shown in Fig. 3. IBMX, a phosphodiesterase inhibitor [5], enhanced glycerol release and $[^3H]TPMP^+$ efflux over the same concentration range. The cAMP analogue, Bt_2cAMP, produced a concentration-dependent increase in glycerol release and $[^3H]TPMP^+$ efflux, whereas Bt_2cGMP (2 mM) or *n*-butyric acid (5–100 μM) had no effect on either parameter *(data not shown)*. Half-maximal glycerol release and $[^3H]TPMP^+$ efflux were observed in response to

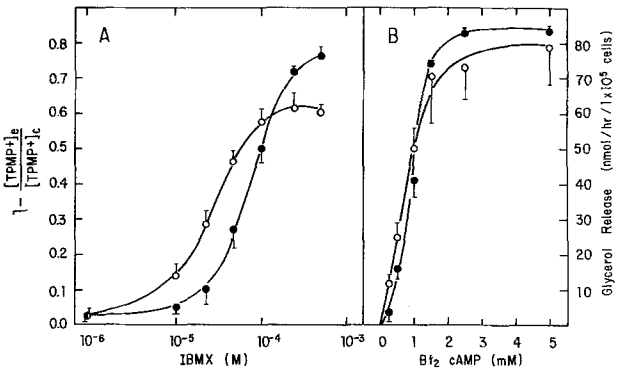

Fig. 3. Effects of IBMX and Bt_2cAMP on $[^3H]TPMP^+$ distribution and lipolysis. Cells were incubated with cAMP-mimetic compounds or vehicle for 60 min at 37 °C in Tris HCl buffer. $[^3H]TPMP^+$ distribution (●): glycerol release above basal (○). All points represent the mean ± SEM of at least three experiments

27 μM IBMX, 750 μM Bt_2cAMP, 70 μM IBMX and 800 μM Bt_2cAMP, respectively.

The kinetics of $[^3H]TPMP^+$ efflux and glycerol release from the adipocyte in response to cholera toxin or ACTH are shown in Figs. 4 and 5. Cholera toxin (1 μg/ml), which activates adipocyte adenylate cyclase after a lag period of ≃1 hr [17, 51] produced a delayed increase in $[^3H]TPMP^+$ efflux and a concomitant increase in glycerol release. The earliest detectable response to ACTH (50 nM) was an increase in intracellular cAMP generation, which peaked at 2 min and declined thereafter to a steady level that was maintained throughout the remainder of the incubation. Glycerol release and $[^3H]TPMP^+$ efflux from the cell were significant after ≃15 min of exposure to ACTH. A similar kinetic profile was obtained with epinephrine *(data not shown)*. These results suggest that the efflux of $[^3H]TPMP^+$ from the adipocyte in response to lipolytic hormones is distal to activation of adenylate cyclase. Also, the

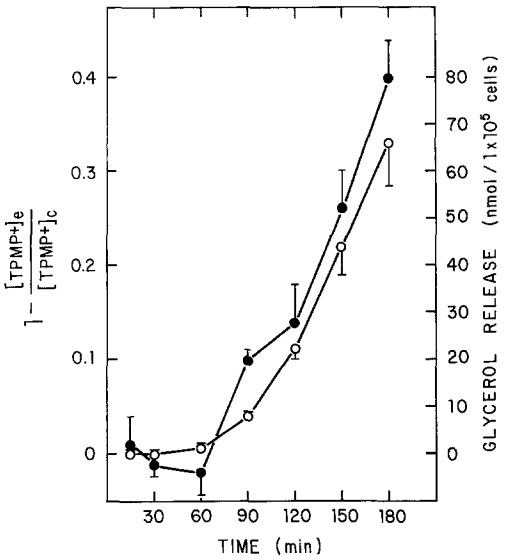

Fig. 4. Effect of cholera toxin on [^{3}H]TPMP$^+$ distribution and lipolysis. Cells were incubated with cholera toxin (1 μg/ml) or vehicle for the indicated times at 37 °C in Tris HCl buffer. [^{3}H]TPMP$^+$ distribution (●): glycerol release above basal (○). All points represent the mean ±SEM of two separate experiments

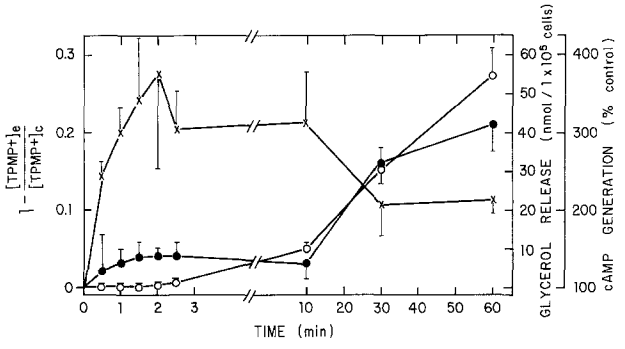

Fig. 5. Effects of ACTH on [^{3}H]TPMP$^+$ distribution, intracellular cAMP generation and lipolysis. Cells were pre-incubated (37 °C) in Tris HCl buffer with [^{3}H]TPMP$^+$ for 30 min so that probe equilibration was achieved prior to the addition of ACTH (50 nM) or vehicle. Samples were withdrawn for determination of [^{3}H]TPMP$^+$ distribution (●): glycerol release above basal (○): % intracellular cAMP generation (×) at the indicated times. All points represent the mean ±SEM of at least three separate experiments

Table 3. Effects of increasing $[K^+]_o$ on TPMP$^+$ distribution and lipolysis in response to Bt_2cAMP

$[KCl]_o$ (mM)	$1-\frac{[TPMP^+]_e}{[TPMP^+]_c}$	Glycerol release (nmol/hr/10^5 cells)
4.8	0.32±0.10	59.3±17.9
10.0	0.23±0.01	63.0±14.4
50.0	0.32±0.02	62.8±13.4
75.0	0.37±0.05	66.7±14.6
100.0	0.34±0.01	65.0±11.2

Cells were incubated with 750 μM Bt_2cAMP or vehicle for 60 min at 37 °C in Tris HCl buffer containing the indicated $[K^+]_o$'s (replacing $[Na^+]_o$). The fraction of TPMP$^+$ that was lost from the cells incubated with Bt_2cAMP at each $[K^+]_o$ is expressed as $1-\frac{[TPMP^+]_e}{[TPMP^+]_c}$. Lipolysis in response to Bt_2cAMP is expressed as glycerol release above basal (basal release was 3–7 nmol/hr/10^5 cells at all $[K^+]_o$'s) at each of the $[K^+]_o$'s. All data represent the mean ±SEM for two separate determinations.

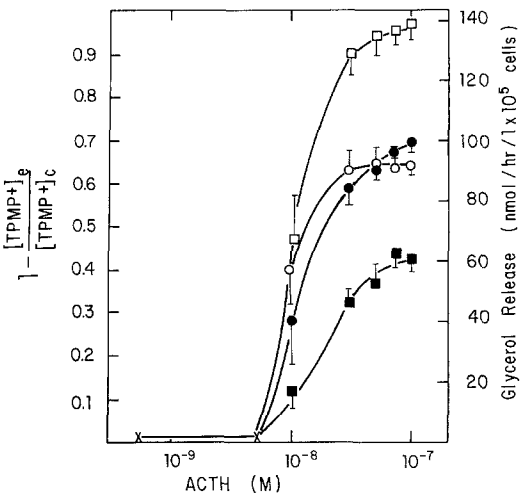

Fig. 6. Effects of $[BSA]_o$ on ACTH-stimulated [^{3}H]TPMP$^+$ distribution and lipolysis. Cells were incubated with ACTH or vehicle for 60 min at 37 °C in Tris HCl buffer containing either 1 or 3% (wt/vol) $[BSA]_o$. [^{3}H]TPMP$^+$ distribution - 1% $[BSA]_o$ (●) or 3% $[BSA]_o$ (■): glycerol release above basal - 1% $[BSA]_o$ (○) or 3% $[BSA]_o$ (□). × indicates the convergence of points at the abscissa that are not significantly different from control. All points represent the mean ±SEM of at least three separate experiments

hormone-stimulated adipocyte depolarization does not appear to be an early, obligatory event in hormonal transduction across the adipocyte plasma membrane.

IV. Subcellular Localization of Bt_2cAMP-induced [^{3}H]TPMP$^+$ Efflux

The possibility that lipolytic compounds produce [^{3}H]TPMP$^+$ efflux from the adipocyte by depolarizing the mitochondrial membrane was examined by incubating cells with Bt_2cAMP under conditions where ψ_p was abolished. The plasma membrane component of ψ_t is essentially eliminated when 100 mM $[Na^+]_o$ in the buffer is replaced by 100 mM $[K^+]_o$. The remainder of the potential-sensitive probe in the cell reflects ψ_m. The effects of 750 μM Bt_2cAMP on [^{3}H]TPMP$^+$ efflux and glycerol release from the adipocyte in the presence of increasing $[K^+]_o$ are shown in Table 3. At all of the $[K^+]_o$'s tested, the ability of Bt_2cAMP to stimulate

both [^{3}H]TPMP$^+$ efflux and glycerol release was unaltered. These data indicate that Bt_2cAMP elicits efflux of the probe via depolarization of the mitochondrial inner membrane and not the plasma membrane, and that the maintenance of ψ_p is not critical for the demonstration of either response. It is likely that ACTH and 1-epinephrine produce probe efflux by the same mechanism as Bt_2cAMP. When cells were exposed to 50 nM ACTH, a concentration that produced maximal glycerol release, at several $[K^+]_o$'s, similar results were obtained. However, a reduction in both glycerol release and TPMP$^+$ efflux in elevated $[K^+]_o$ buffer compared to normal buffer was observed when submaximal concentrations of ACTH or 1-epinephrine were utilized *(data not shown)*. These experiments are complicated by the fact that hormone-receptor binding on the plasma membrane is voltage and/or ion dependent [8, 16, 52] and that other hormonal transduction events, *i.e., coupling* may be regulated by plasma membrane potential.

V. Effects of $[BSA]_o$ on [^{3}H]TPMP$^+$ Efflux and Glycerol Release

The effects of $[BSA]_o$ on glycerol release and [^{3}H]TPMP$^+$ efflux from the adipocyte in response to increasing concentrations of ACTH are shown in Fig. 6. When $[BSA]_o$ was increased from 1 to 3% (wt/vol), ACTH-stimulated glycerol release was enhanced whereas [^{3}H]TPMP$^+$ efflux was diminished. Similar results were obtained with epinephrine and Bt_2cAMP. In control experiments, there was no significant binding between [^{3}H]TPMP$^+$ and BSA. Moreover, exogenous glycerol (10–600 nmol) had no effect on [^{3}H]TPMP$^+$ distribution into the adipocyte *(data not shown)*.

Serum albumin provides binding sites for FFA that are released from the cell in response to lipolytic compounds [21]. When the molar ratios of medium FFA/albumin exceed 3:1, adenylate cyclase and triglyceride lipase are inhibited [20, 34], suggesting that FFA serve as negative feedback regulators of lipolysis. Inhibition of lipolysis may result from a direct interaction between FFA and lipase [21]. Alternatively, FFA may indirectly inhibit lipolysis by limiting ATP availability and/or increasing cytosolic calcium levels via de-energization of the mitochondrial inner membrane since both the ADP-phosphorylating and calcium-accumulating capacities of mitochondria are dependent upon energization of the inner membrane [47, 48]. Lipolytic enzymes require ATP and a reduction in cytosolic ATP levels and/or an increase in free cytosolic calcium levels could inhibit their activity [31, 40].

The observed effects of $[BSA]_o$ on glycerol release and [^{3}H]TPMP$^+$ efflux can be explained on the following basis. Increasing the $[BSA]_o$ from 1 to 3% provides a substantial number of additional binding sites for the FFA that are generated in response to ACTH. Experimental verification for the saturation of BSA binding sites by FFA was obtained by estimating the molar ratio of FFA/BSA under the different incubation conditions [*refer to* Appendix]. That is, less FFA will accumulate intracellularly in the buffer containing 3% BSA at a given concentration of ACTH. As a result, there will be less depolarization of the mitochondrial inner membrane and less probe efflux in the buffer containing 3% $[BSA]_o$. Furthermore, since FFA may serve as negative feedback regulators of lipolysis by reducing ψ_m, glycerol release will be greater in the buffer containing 3% $[BSA]_o$ compared to the buffer containing 1% $[BSA]_o$.

Several lines of experimental evidence support this hypothesis. Lipolytic hormones reduce adipocyte ATP levels and increase respiration in a manner that is not tightly coupled to phosphorylation [24]. There is a direct correlation between the reduction in ATP levels and the intracellular accumulation of FFA [1, 2]. In isolated mitochondria, FFA uncouple oxidative phosphorylation, dissipate the mitochondrial protonmotive force and increase calcium efflux [4, 43].

Mitochondrial uncouplers, ionophores and oligomycin reduce cytosolic ATP levels and inhibit hormone-stimulated activation of adenylate cyclase and lipolysis [22, 28, *unpublished observations*]. According to the data shown in Table 1, they also produce [^{3}H]TPMP$^+$ efflux from the cell presumably by depolarizing the inner mitochondrial membrane. These observations implicate the mitochondria as having a role in the lipolytic responsiveness of the adipocyte.

The possibility that intracellular FFA accumulation in response to lipolytic hormones serves as a negative feedback regulator of lipolysis in adipocytes via a direct effect on the mitochondrial membrane potential deserves further consideration. Studies of this nature may provide valuable information concerning homeostatic regulatory mechanisms in the intact cell.

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Appendix

A. Calculation of the Relative Amount of $TPMP^+$ that Partitions Into the Rat White Adipocyte

Cells were incubated at 37 °C in Tris HCl buffer for 60 min (data from a typical experiment are presented).

Given: (a) 2,196,800 cpm's $TPMP^+$/1 ml sample
(b) 41,940 cpm's $TPMP^+$ was cell-associated/1 ml sample
(c) 290,000 adipocytes/1 ml sample.

Thus, 1.9% of the $TPMP^+$ was associated with the cells in a sample.

B. Calculation of Adipocyte Plasma Membrane Potential (ψ_p) and Mitochondrial Membrane Potential (ψ_m)

The plasma and mitochondrial membrane potentials can be estimated according to the transmembrane distribution ratio of $[TPMP^+]$ in the presence and absence of 5 μM CCCP. Adipocyte intracellular volumes were obtained from [18] or [41] using the appropriate conversion factors to normalize for the number of cells present in a sample. The size of the rats used in [18], [41] and in the present experiments were similar, so that the H_2O spaces should be comparable.

Cells were incubated with 1 μM $[TPMP^+]_o$ at 37 °C in Tris HCl buffer ±5 μM CCCP for 60 min (data from a typical experiment are presented).

Given: (a) $[TPMP^+]_o = 1$ μM
(b) 290,000 adipocytes/ml sample.

Assumptions:
(a) Intracellular volume
1. 0.38 μl H_2O/290,000 cells [18] or
2. 0.55 μl H_2O/290,000 cells [41].
(b) mitochondrial volume ≙ 2% intracellular volume [18].
(c) $[TPMP^+]_{cytoplasm}$ = remaining cell-associated $TPMP^+$ in the presence of CCCP
(d) $[TPMP^+]_{mitochondria}$ = total cell-associated $TPMP^+$ minus $[TPMP^+]_{cytoplasm}$
(e) $$\psi = -61.5 \log \frac{[TPMP^+]_{outside}}{[TPMP^+]_{inside}}$$

1. (a) $[TPMP^+]_{cytoplasm} = 4.5$ μM using the adipocyte intracellular volume as determined in [18].
$$\psi_p = -61.5 \log \frac{[1.0\ \mu M\ TPMP^+]_o}{[4.5\ \mu M\ TPMP^+]_i}$$
$$\psi_p = -40.2\ mV$$
(b) $[TPMP^+]_{mitochondria} = 2293.4$ μM
$$\psi_m = -61.5 \log \frac{[4.5\ \mu M\ TPMP^+]_{cytoplasm}}{[2293.4\ \mu M\ TPMP^+]_{mitochondria}}$$
$$\psi_m = -166.5\ mV.$$

In addition, ψ_m can be calculated when $\psi_p = 0$ (when cells are incubated in 120 mM $[K^+]_o$). Under these conditions, the amount of probe that remains associated with the cells equals the $TPMP^+$ inside the mitochondria plus the potential-insensitive cytoplasmic $TPMP^+$ (1 μM $[TPMP^+]_{cytoplasm}$, when $\psi_p = 0$).

ψ_m = total $TPMP^+$-potential-insensitive cytoplasmic $TPMP^+$

(c) $[TPMP^+]_{mitochondria} = 782.9$ μM
$$\psi_m = -61.5 \log \frac{[1.0\ \mu M\ TPMP^+]_{cytoplasm}}{[782.9\ \mu M\ TPMP^+]_{mitochondria}}$$
$$\psi_m = -178.0\ mV$$

2. Similarly, ψ_p and ψ_m can be estimated from the cytoplasmic volume as determined in [41]. The values for ψ_p and ψ_m compare reasonably well using either estimate of adipocyte intracellular volume.

(a) $$\psi_p = -61.5 \log \frac{[1.0\ \mu M\ TPMP^+]_o}{[3.1\ \mu M\ TPMP^+]_i}$$
$$\psi_p = -30.2\ mV$$
(b) $$\psi_m = -61.5 \log \frac{[3.1\ \mu M\ TPMP^+]_{cytoplasm}}{[1584.5\ \mu M\ TPMP^+]_{mitochondria}}$$
$$\psi_m = -166.7\ mV$$
(c) $$\psi_m = -61.5 \log \frac{[1.0\ \mu M\ TPMP^+]_{cytoplasm}}{[540.9\ \mu M\ TPMP^+]_{mitochondria}}$$
$$\psi_m = -168.1\ mV.$$

C. Calculation of medium FFA/$[BSA]_o$

Cells were incubated at 37 °C in Tris HCl buffer containing 1% $[BSA]_o$ or 3% $[BSA]_o$. After 60 min, ACTH-stimulated glycerol release was measured. The values reported represent the maximal amount of glycerol that was released by ACTH (50 nM). Data from three experiments are presented as mean glycerol release (basal + hormone-stimulated).

1% $[BSA]_o$ = 166.6 nmol BSA/sample and 205.0 nmol glycerol/sample.
3% $[BSA]_o$ = 500.1 nmol BSA/sample and 296.3 nmol glycerol/sample.

Assumption: 3 FFA released/1 glycerol released.

In 1% $[BSA]_o$ buffer, the FFA/BSA ≃ 3.7/1.0.
In 3% $[BSA]_o$ buffer, the FFA/BSA ≃ 1.8/1.0.

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